Regulation of Myometrial Contractility: Defining the Contribution of the MaxiK Potassium Channel and the L- and T-type Calcium Channels.

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2015

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Acknowledgements

I started this PhD as I enjoyed working in the lab and I wanted to challenge myself to see just what I was capable of achieving. Four years later I have realised that scientific research is the only career in the world for me. I have loved doing this PhD, the challenge of getting an experiment to work, the anticipation as I have analysed the results and that bubbling excitement as I realise I am making a connection no-one has made before. None of this would have been possible without the support of my supervisor, Neil. So I want to thank him for his belief in my abilities, and for having enough faith in me to allow me to take control of my PhD and to follow my theories through to their conclusion even when they sounded a little unconventional! Honestly, I have not got the words to express just how grateful I am to Neil for everything he has done over the last four years – So I'll just say thank you for being with me on this roller coaster ride and here's to the future whatever it may hold.

I also want to thank Danièle, Elspeth, Katrina, and Mark who have spent countless hours listening to me, and allowing me to bounce ideas off them. Thank you to Mark for your steady supply of emergency coffee! Elspeth for your surprise food packages, Katrina and Danièle for keeping me sane! Without their input and support there are many ideas in this thesis that wouldn't have made it out of my head and onto the lab bench! Thanks also go to the Ellen Webster foundation for funding my studies.

I couldn't finish this section without a special mention for my boys, Andrew and Michael, they are simply the most amazing little people. Looking at them each day reminds me of just how important the work in this thesis is – I want everyone to have the chance to experience the joy of watching their own healthy happy children grow and develop. Andrew and Michael you are Mum's little rays of sunshine. Your hugs on the days when I got home frustrated made all the frustration worthwhile and your surprise when I showed you my work and you exclaimed "Mummy, do you just draw pictures all day?" has kept me grounded. You are my reason for being and my reason for doing this. If you can learn just one thing from my doing this PhD it should be that you can achieve pretty much anything with hard work and determination.

Publications Arising from this Thesis

Waite, S. L., Gandhi, S. V., Khan, R. N. and Chapman, N. R. (2014) The effect of trichostatin-A and tumor necrosis factor on expression of splice variants of the KCNMA1 and CACNA1C channels in human myometrium. Front Physiol. **5**:261.

Publications Associated with this Thesis

Webster, S. J., **S. L. Waite**, V. J. Cookson, A. Warren, R. Khan, S. V. Gandhi, G. N. Europe-Finner and N. R. Chapman (2013). "Regulation of GTP-binding protein (Gαs) expression in human myometrial cells: a role for tumor necrosis factor in modulating Gαs promoter acetylation by transcriptional complexes." J Biol Chem **288**(9): 6704-6716.

Cookson, V. J.[*], **S. L.**[*] **Waite**, P. R. Heath, P. J. Hurd, S. V. Gandhi, and N. R. Chapman (2015). "Binding Loci of RelA-containing Nuclear factor-kappaB (NF-κB) Dimers in Promoter Regions of PHM1-31 Myometrial Smooth Muscle Cells." Mol. Hum. Reprod. **21**(11): 865-883
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Abstract

This thesis describes a comprehensive study investigating the roles of the MaxiK potassium channel (KCNMA1), L-Type calcium channel (CACNA1C) and T-Type calcium channel (CACNA1G) in the maintenance of quiescence (relaxed myometrium), the preparation for parturition (non-contracting myometrium) and the regulation of the co-ordinated contractions characteristic of parturition itself (contracting myometrium). The role of these channels was investigated using primary human myometrial cell cultures under relaxed, non-contracting and contracting conditions. Protein studies revealed changes in both the amount and channel isoforms expressed between the different conditions. Protein-protein interaction studies revealed that the KCNMA1 and CACNA1C associated with Caveolin-1, $G_{\alpha s}$ and β_2-Adrenergic Receptor. RNA studies revealed that the different incubation conditions modified expression of total channel mRNA and that of various splice variants. Previous research has demonstrated that the CACNA1C channel C-terminus can function as a transcription factor termed CCAT. Within this thesis inmmunohistochemistry staining and protein localisation studies revealed nuclear localisation of both the CACNA1C and KCNMA1 C-terminii. Therefore, genomic studies were undertaken utilising the ChIP assay, coupled with ChIP sequencing, to study the role of the KCNMA1 channel as a transcription factor. Chip-sequencing data files were then analysed using Galaxy, an open access web-based platform. Peak calling generated 47 peaks, 21 were successfully mapped to known genes, including RB1, JPH2 and MAP3K7. Motif discovery was then undertaken for both the KCNMA1 protein utilising GYM and the successfully mapped peaks using the Panoptic Motif Search Tool. A helix-turn-helix motif was discovered in the C-terminal region of the KCNMA1 protein and ten putative transcription factor binding motifs were discovered within the peak regions. The significance of these findings is discussed.

Abbreviations

aa	Amino Acid
AC	Adenylate Cyclase
ACTA2	Actin α2
ADP	Adenosine Diphosphate
AGE	Agarose Gel Electrophoresis
AID	α-Interaction Domain
ATP	Adenosine Triphosphate
bp	Base Pair
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium Ions
CACNA1C	CACNA1C calcium channel
CACNA1G	CACNA1G calcium channel
cAMP	Cyclic Adenosine Monophosphate
Cav1	Caveolin 1
CBP	CREB Binding Protein
CCAT	Calcium Channel Associated Transcription Factor
cGMP	Cyclic Guanosine Monophosphate
ChIP	Chromatin Immunoprecipitation
ChIP-seq	ChIP sequencing
CNS	Central Nervous System
COX-2	Cyclo-Oxygenase 2
CpG	Cytosine – phosphate – Guanine
CREM	cAMP-Response Element Modulator
DAG	1,2-Diacylglycerol
dATP	2'-Deoxyadenosine 5'-Triphosphate
dCTP	2'-Deoxycytidine 5'-Triphosphate
DEPC	Diethylpyrocarbonate
dGTP	2'-Deoxyguanosine 5'-Triphosphate
DHP	Dihydropyridine
DMEM	Dulbeccos, Modified Eagles' Medium
DMSO	Dimethy Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
DSS	Disuccinimidyl Suberate
dTTP	2'-Deoxythymidine 5'-Triphosphate
ECL	Enhanced Chemiluminesence
ECM	Extracellular Maxtrix
EDTA	Ethylenediamine Tetraacetic Acid
Egr-1	Early Growth Response Protein 1
EGTA	Ethylene Glycol Tetraacetic Acid
EMSA	Electron Mobility Shift Assay
ENCODE	Encyclopedia of DNA Elements
EP	Prostaglandin E Receptor
ER	Endoplasmic Reticulum
ESE	Exonic Splicing Enhancer
FAT	Factor Acetyltransferase
FCS	Foetal Calf Serum
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GATA-6	GATA Binding Protein 6
GTP	Guanosine-5'-Triphosphate
$G_{\alpha i}$	G_i Alpha Subunit
$G_{\alpha q}$	G_q Alpha Subunit
$G_{\alpha s}$	G_s Alpha Subunit
Gβ	G Protein Beta Subunit
hCG	Human Chorionic Gonadotropin
HDAC	Histone Deacetylase

HEK	Human Embryonic Kidney
hnRNPA1	Heterogeneous Nuclear Ribonucleoprotein A1
HPA axis	Hypothalmic-pituitary -adrenal axis
HRP	Horse Radish Peroxidase
HTH	Helix-Turn-Helix
HUVEC	Human Umbilical Vein Endothelial Cells
IKK	IκB Kinase
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IL-8	Interleukin 8
IP	Immunoprecipitation
IP3	Inositol 1,4,5-Triphosphate
IκBα	Nuclear Factor of Kappa Light Chain Polypeptide Gene Enhancer in B-cells Inhibitor, Alpha
JPH2	Juncophillin 2
KCl	Potassium Chloride
KCNMA1	Large Conductance Calcium Activated Potassium Channel
KCNMB3	KCNMA1 β subunit
kDa	Kilodalton
LH	Luteinizing Hormone
LPS	Lipopolysaccharide
CACNA1C	CACNA1C Calcium Channel
MAP3K7	Mitogen Activated Protein Kinase Kinase Kinase 7
KCNMA1/slo-1 / BK	Large Conductance Calcium Activated Potassium Channel
MeDIP	Methylated DNA Immunoprecipitation
MLCK	Myosin Light Chain Kinase
MLCP	Myosine Light Chain Phosphatase
MMP	Matrix Metalloproteinase
modENCODE	Model Organism ENCODE
MQ	MilliQ
MZF	Myeloid Zinc Finger Protein
n/s	Non Specific
NaOH	Sodium Hydroxide
NCBI	National Center for Biotechnology Information
NFAT	Nuclear Factor of Activated T-cells
NFκB	Nuclear Factor Kappa B
NGS	Next Generation Sequencing
NIK	Nf-kappab-Inducing Kinase
NP40	Nonident P-40
OR	Oxytocin Receptor
p65	RelA
PBS	Phosphate Buffered Saline
PBS-T	PBS-tween 20
PCR	Polymerase Chain Reaction
PF	Passing Filter
PGE2	Prostaglandin E2
PGF2α	Prostaglandin F2 Alpha
PIP2	Phosphatidylinositol 4,5-Bisphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PKG	Protein Kinase G
PLC	Phospholipase C
pM	Pico Mole
PMCA	Plasma Membrane Calcium ATPase Transporter
PP2A	Protein Phosphatase 2
PR	Progesterone Receptor
QC	Quality Control
RB1	Retinoblastoma Protein
RCK	Regulator of Conductance of K^+ domain

RelA	v-rel avian reticuloendotheliosis viral oncogene homolog A / NFκB p65
RNA	Ribonucleic Acid
rpm	Revolutions Per Minute
SA	Sino-Atrial
SCIER	Spatial Clustering for Identification of ChIP Enriched Regions
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
SERCA	Sarco/Endoplasmic Reticulum Calcium ATPase Transporter
SF2	Splice Factor 2
SITraN	Sheffield Institute for Translational Neuroscience
SP1	Specificity Protein 1
SP-A	Surfactant Protein A
SR	Sarcoplasmic Reticulum
STREX	Stress Regulated Exon
TAE	Tris Acetate EDTA
TBS	Tris Buffered Saline
TBS-T	TBS-Tween 20
TBS-TM	Membrane Blocking Buffer Milk
TF	Transcritpion Factor
TGB	Tris Glycine Buffer
Thy-1	Thymocyte Antigen 1
TLR	Toll-like Receptor
TLR2	Toll-like Receptor 2
TLR4	Toll-like Receptor 4
TNF	Tumour Necrosis Factor
TSA	Trichostatin A
TSS	Transcription Start Site
TTB	Towbin Transfer Buffer
CACNA1G	CACNA1G Calcium Channels
v/v	Volume/Volume
w/v	Weight/Volume
WCL	Whole Cell Lysis
WGA	Whole Genome Amplification
β_2AR	Beta 2 Adrenergic Receptor

Chapter 1 Introduction

1.1 Reproduction

Reproduction is a fundamental process which occurs in all living organisms. It is arguably the most important, as successful reproduction ensures the survival of the species. In mammals, successful reproduction includes the distinct processes of: fertilisation, implantation, maturation (pregnancy) and birth (parturition). Despite the critical importance of these processes our understanding of them is still limited, especially in terms of describing the regulation of the switch between the pregnant state and parturition.

1.1.1 Parturition as a Controlled Inflammatory Event

Traditionally, parturition was thought to be a hormonal event driven predominantly by changes in progesterone or cortisol synthesis. This thesis was based on strong evidence from animal models; however, human parturition does not reflect such models (Golightly, Jabbour *et al.* 2011). This has lead to a number of theories regarding the regulation of human parturition. Firstly, it has been suggested that multiple paracrine/autocrine events, fetal hormonal changes and overlapping maternal/fetal control mechanisms are responsible for triggering parturition. Alternatively, there is growing evidence to suggest that the process of human parturition is a controlled inflammatory event coupled with a functional progesterone withdrawal (see Section 1.2.5 *Progesterone Withdrawal*). At the onset of physiological parturition there is an influx of pro-inflammatory cytokines which coincides with an influx of inflammatory cells, neutrophils and macrophages, in both the upper and lower myometrium (Thomson, Telfer *et al.* 1999, Osman, Young *et al.* 2003, Golightly, Jabbour *et al.* 2011). These inflammatory cytokines and cells are significantly reduced in women undergoing elective Caesarean section in comparison to women undergoing term parturition. This demonstrates the role these factors have in term parturition. This inflammation is not caused, in the majority of cases, by infection, but instead is a form of sterile inflammation (Gomez-Lopez, Guilbert *et al.* 2010, Kobayashi 2012). However, a study examining the presence of inflammation in uterine and gestational tissues found that inflammation was rare in these tissues prior to the onset of parturition (Keski-Nisula, Aalto *et al.* 2000). This would seem to suggest that inflammation is a consequence of parturition as opposed to the cause of parturition.

1.1.2 Sterile Inflammation

Whereas non-sterile inflammation is a response to pathogens, sterile inflammation occurs as a response to physical, chemical or metabolic noxious stimuli. Each stimulus produces a tightly regulated stress response. These stress responses induce the recruitment of inflammatory cells such as neutrophils, macrophages and leukocytes and result in inflammation (Rubartelli, Lotze *et al.* 2013).

Preceding parturition there are a number of events which could initiate or enhance this sterile inflammation pathway. Firstly, towards the end of pregnancy the myometrium is stretched by the growing fetus, this mechanical stretch induces an increase in the secretion of pro-inflammatory cytokines which could initialise or promote sterile inflammation (Kobayashi 2012). Secondly, the process of cervical ripening which involves the degradation of extracellular matrix proteins also leads to the release of molecules that activate inflammatory signalling pathways and could enhance the sterile inflammation process (Christiaens, Zaragoza *et al.* 2008, Challis, Lockwood *et al.* 2009). Finally, the concentration of pro-inflammatory cytokines found in amniotic fluid increases towards parturition. These pro-inflammatory cytokines have been shown to induce chemotaxis of neutrophils, macrophages and leukocytes into the uterus. Once in the uterus, these inflammatory cells then release cytokines, matrix metalloproteinases (MMPs), prostaglandins and more chemokines (Gomez-Lopez, Guilbert *et al.* 2010). This then amplifies inflammatory signalling and could again enhance the sterile inflammation process.

1.2 Regulation of These Parturition Events

The contractions seen at parturition is the culmination of a process involving fetal maturation, membrane rupture, placental separation, cervical ripening and dilation which then ultimately lead to co-ordinated uterine contractions (Lindstrom and Bennett 2005). Over the last few years a significant body of research has been undertaken to define these events leading up to, and including, parturition.

The process of parturition is characterised by an increase in pro-inflammatory cytokines such as Interleukin 1 beta (IL-1β), Interleukin 6 (IL-6) and Tumour Necrosis Factor (TNF) (Romero, Mazor *et al.* 1992, Opsjln, Wathen *et al.* 1993, Keelan, Marvin *et al.*

1999, Osman, Young *et al.* 2003). These cytokines are thought to enhance the production of Interleukin 8 (IL-8) and prostaglandins, which then work in concert to promote cervical ripening and ultimately, uterine contractions. However, the induction of this inflammatory signalling cascade is still to be fully elucidated.

There are also signalling cascades generated from both the fetal and the endocrine systems. Briefly, the fetus has been shown to co-ordinate parturition associated changes in the myometrium via its influence on placental steroid hormone production. Alongside this there is a shift from progesterone to estrogen predominance, increased myometrial oxytocin receptor and prostaglandin expression and GAP junction formation. These changes are followed by activation of the fetal hypothalamic-pituatry-adrenal (HPA) axis and ultimately parturition (Kota, Gayatri et al. 2013; Figure 1.1).

There have been a number of suggestions as to the key events which regulate the initiation of these signalling cascades including: secretion of surfactant protein A (SP-A) as a signal of fetal lung maturity, initiation of membrane rupture and cervical ripening through increased MMP expression, prostaglandin synthesis promoting myometrial contractions, transcriptional regulation by Nuclear Factor kappa B (NFκB) promoting the expression of pro-contractile genes, functional progesterone withdrawal resulting in the removal of the support for quiescence, and regulation of ion channel expression to increase the contractility of the myometrium (Figure 1.1). These are discussed below.

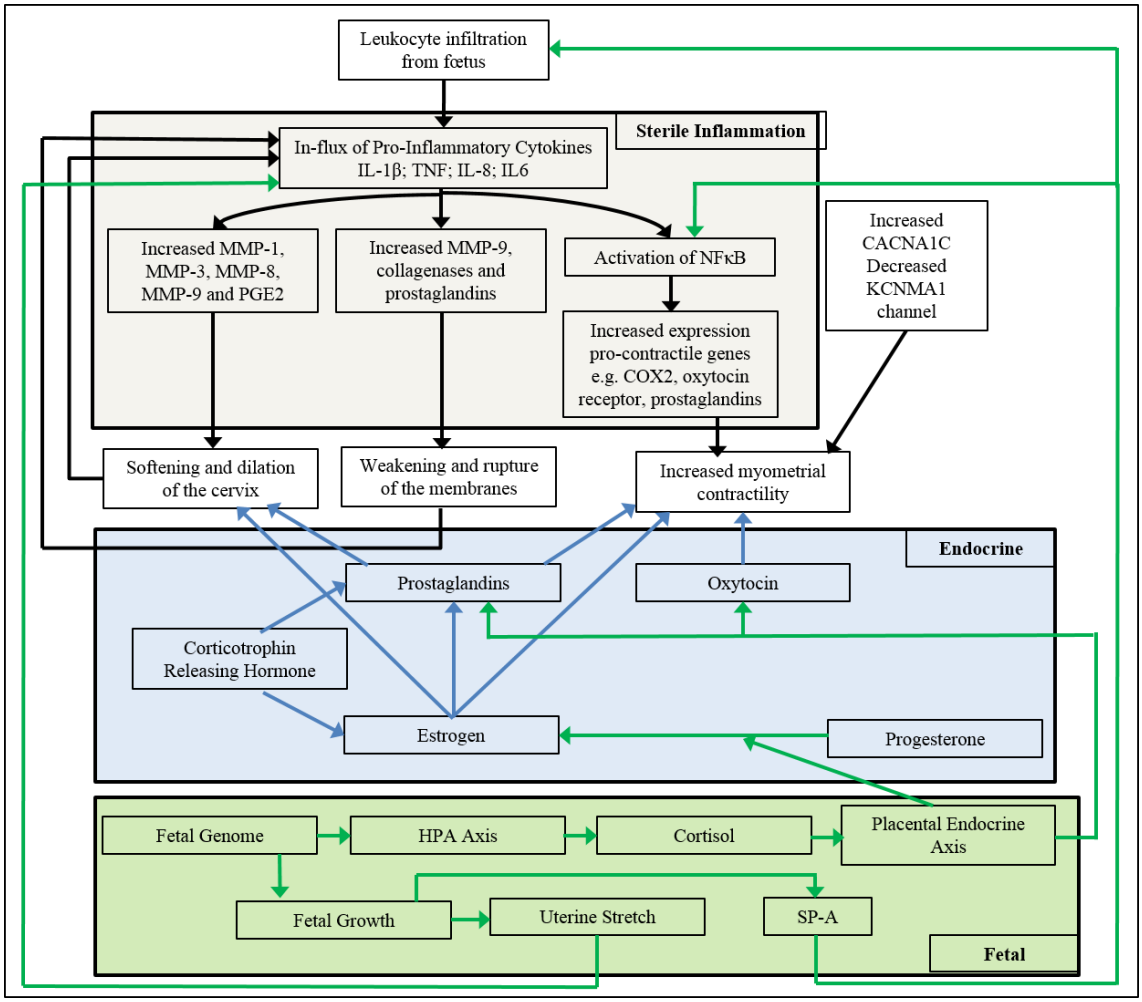


Figure 1.1 Regulation of Parturition

A summary of the current understanding regarding the regulation of the processes of cervical ripening, membrane rupture and myometrial contractility. The sterile inflammation process is within the grey shaded box. Briefly, leukocyte infiltration from the fetus leads to an influx of proinflammatory cytokines. The increase in pro-inflammatory cytokines alongside an increase in SP-A leads to the activation of MMPs, Protaglandins, collagenases and NFκB. These, alongside an increase in CACNA1C and a decrease in KCNMA1 expression, subsequently lead to softening and dilation of the cervix, rupture of the fetal membranes and increased myometrial contractility (black arrows). The endocrine process is within the blue shaded box. Briefly, there is a switch to estrogen predominance leading to increased prostaglandins synthesis alongside increased oxytocin responsiveness again leading to softening and dilation of the cervix and increased myometrial contractility (blue arrows). Fetal factors are within the green shaded box. Briefly, activation of the fetal Hypothalmic-pituitary-adrenal (HPA) axis leads to activation of the placental endocrine axis which promotes the switch to estrogen predominance and the increased expression of prostaglandins and oxytocin responsiveness. Alongside this fetal growth produces uterine stretch and SP-A production which can then activate the sterile inflammation pathway. Both again lead to softening and dilation of the cervix, rupture of the fetal membranes and increased myometrial contractility (green arrows).

1.2.1 The Role of Surfactant Protein A (SP-A)

SP-A is secreted by the fetal lungs and is used as a marker of fetal lung maturity (King, Ruch *et al.* 1975, Hallman, Arjomaa *et al.* 1988, Miyamura, Malhotra *et al.* 1994). Levels of SP-A peak at term and it is thought they may provide a fetal signal for the commencement of parturition (Mendelson and Condon 2005, Mendelson 2009).

Increased SP-A induces the activation of NFκB and production of IL-1β from fetal macrophages, which in turn migrate to the uterus and cause an inflammatory response and an increase in uterine NFκB (Condon, Jeyasuria et al. 2004; Figure 1.1). Injection of the amniotic sacs of mice 15 days post coitum with 6μg SP-A was seen to induce nuclear localisation of the NFκB RelA subunit and preterm parturition (Condon, Jeyasuria *et al.* 2004, Lindstrom and Bennett 2005, Garcia-Verdugo, Tanfin *et al.* 2008). These findings suggest that the signal for the initiation of parturition is the secretion of SP-A, which then causes the migration of macrophages into the uterus, followed by the secretion of pro-inflammatory cytokines. These pro-inflammatory cytokines then activate NFκB which in turn promotes the transcription of pro-contractile genes such as Cyclo-oxygenase 2 (Cox-2) leading to contractions. It is important to note however, that NFκB can also be activated by cellular stress (Hoesel and Schmid 2013). Therefore, the increase in nuclear localisation observed in the above studies may be as a response to stress rather than SP-A

In addition to its role in regulating fetal macrophage migration into the uterus, SP-A is also a ligand for Toll-like receptor 2 (TLR2; (Sato, Sano et al. 2003, Yamada, Sano et al. 2006, Henning, Azad et al. 2008) and Toll-like receptor 4 (TLR4; (Henning, Azad et al. 2008). Toll-like receptors (TLRs) are a family of membrane bound proteins that recognise pathogens and as such binding of TLRs constitutes the initial event in the activation of the immune response. Binding of TLRs also results in the activation of NFκB and the initiation of an inflammatory cascade. A recent study into the interaction between SP-A and both TLR2 and TLR4 made the surprising discovery that when SP-A is administered into the uterine cavity it supressed both inflammation and parturition (Agrawal, Smart *et al.* 2013). This would appear to refute its role in the initiation of parturition as this research has shown SP-A can supress parturition.

A further mechanism by which surfactant proteins may regulate the timing of parturition is through their role in the synthesis of prostaglandins. Surfactant protein is secreted from the fetal lung into the amniotic fluid, this surfactant protein is then responsible for the release of arachidonic acid which is the precursor for prostaglandin synthesis (Newman, Phizackerley *et al.* 1993, Bernal and Phizackerley 2000, Nayak, Dodagatta-Marri *et al.* 2012). Briefly, arachidonic acid is cleaved from a phospholipid by phospholipase. Within the amninon, acrachidonic acid is synthesised by the cleavage of amniocyte diacyl

phosphatidylethanolamine by phospholipase A2 or phosphatidylinositol by phospholipase C and diacylglycerol lipase. Following this, arachidonic acid is converted to prostaglandin G_2 by cyclooxygenase enzymes. Prostaglandin G_2 is then converted to prostaglandin H_2 by the peroxidase activity of the cyclooxygenase enzymes. Finally, prostaglandin H_2 is converted by different prostanoid synthases to various prostanoids including prostaglandin E_2 and prostaglandin $F_{2\alpha}$ (Gualde and Harizi 2004).

Clearly, the study by Agrawal *et al.* (Agrawal, Smart *et al.* 2013) contradicts the earlier findings by Condon *et al.* that SP-A is involved in pro-inflammatory signalling and can induce preterm parturition (Condon, Jeyasuria *et al.* 2004). This may be due to the fact that in the study by Condon *et al.* SP-A was injected into the amniotic sac whereas in the study by Agrawal *et al.* SP-A was injected into the uterine cavity. It is possible therefore that SP-A has different roles depending on its location. A second possible explanation for these differences is that the study by Agrawal *et al.* specifically examined the role of SP-A in infection induced parturition. In the presence of infection the anti-inflammatory role of uterine SP-A may be important in delaying parturition until the fetus is fully matured at which stage the pro-inflammatory action of SP-A from the amniotic sac initiates parturition. In addition to this the role of surfactant protein in the synthesis of prostaglandins could either lead to a support for contraction or relaxation depending on which prostaglandin receptors are expressed (The role of prostaglandins is more fully discussed in Section 1.2.3 *Prostaglandin Synthesis*). Further work is needed to understand these conflicting roles for SP-A.

1.2.2 Matrix Metalloproteinase (MMP) Expression

The processes of fetal membrane rupture and cervical ripening both involve extensive remodelling of the extracellular matrix (ECM; (Bryant-Greenwood and Yamamoto 1995, Kelly 2002). The ECM of the fetal membranes and decidua is composed primarily of collagen types I, III, IV and V (Aplin, Campbell *et al.* 1985, Leushner and Clarson 1986). Within the cervix the ECM contains collagen types I and III (Minamoto, Arai *et al.* 1987, House, Kaplan *et al.* 2009). Remodelling of the ECM is achieved through a course of programmed collagenolysis which is mediated by MMP enzymes. Collagen degradation is vital in the process of cervical ripening as it causes the cervix to soften and become distensible, which in turn facilitates the passage of the fetus.

It has been shown that during parturition there is a decrease in collagen concentration, an increase in collagen solubility and activation of MMPs (Hampson, Liu *et al.* 1997, Goldman, Weiss *et al.* 2003). At parturition there has been reported to be a significant increase in MMP3 mRNA (41-fold) and protein within the myometrium. MMP3 degrades collagen types I, II and IV as well as fibronectin, laminin and elastin all of which are components of the cervical ECM (Bryant-Greenwood and Yamamoto 1995, O'Brien, O'Shaughnessy *et al.* 2007). This suggests that MMP3 has a significant role in remodelling the cervical ECM in preparation for birth. MMP3 is also able to activate MMP9 (Van den Steen, Dubois *et al.* 2002, O'Brien, O'Shaughnessy *et al.* 2007). MMP9 expression in the fetal membranes has been reported to be significantly increased both at term and preterm parturition (Xu, Alfaidy *et al.* 2002). MMP9 degrades collagen types IV and V which are components of the ECM of both the fetal membranes and decidua (Aplin, Campbell *et al.* 1985, Leushner and Clarson 1986, Delclaux, Delacourt *et al.* 1996). This suggests that MMP9 has a significant role in the ECM degradation cascade leading to membrane rupture. MMP9 has also been implicated as a regulatory factor in neutrophil migration across basement membranes (Delclaux, Delacourt *et al.* 1996). The increase in MMP3 leads to cervical ripening and remodelling of the ECM (Figure 1.1). MMP3 could then activate MMP9 leading to fetal membrane rupture. These events would also enhance the sterile inflammatory state of the myometrium via the release of inflammatory molecules due to the degradation of the ECM and the facilitation of neutrophil migration by MMP9.

1.2.3 Prostaglandin Synthesis

Prostaglandins play a central role in the process of cervical ripening, dilation and stimulation of myometrial contractions. Prostaglandin synthesis has been shown to be a dynamic process which is dependent on both gestational stage and stimulus (Pollard and Mitchell 1996). Spontaneous active labour is associated with a surge of prostaglandin E2 (PGE2) and prostaglandin F2α (PGF2α) which leads to a biochemical cascade similar to a localised acute inflammatory response (Romero, Munoz *et al.* 1996, Brown, Alvi *et al.* 1998). Inhibition of prostaglandins has been utilised to halt the progression of parturition (Besinger, Niebyl *et al.* 1991), while administration of prostaglandins has been shown to induce parturition (Ray and Garite 1992).

PGE2 has been shown to have a diverse set of physiological actions which are dependent on the distribution and subtype of receptors (EP) available. There are four PGE2 receptors (EP_1, EP_2, EP_3, EP_4), all of which are expressed in the myometrium during pregnancy. Both EP_1 and EP_3 can couple to either G protein alpha subunit, group Q ($G_{\alpha q}$) or G protein alpha subunit, group I ($G_{\alpha i}$; Rundhaug, Simper et al. 2011). When PGE2 binds to its EP_1 receptor it initialises a signalling cascade that activates phospholipase C (PLC), resulting in the production of both 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). DAG results in the activation of protein kinase C (PKC) whilst IP3 results in the mobilisation of calcium (Figure 1.2; Rundhaug, Simper et al. 2011). When PGE2 binds to its EP_3 receptor it initialises a separate signalling cascade which blocks the activation of adenylate cyclase (AC), with the effect of reducing adenosine-3',5'-cyclic monophosphate (cAMP) levels. This then reduces the levels of active protein kinase A (PKA), leading to an increase in myosin light chain phosphorylation (Rundhaug, Simper *et al.* 2011) and ultimately an increase in contractility (Figure 1.2). The binding of PGE2 to either of these receptors would promote both contraction and the potentiation of the inflammatory signal. Conversely, EP_2 and EP_4 couple to G protein alpha subunit, group S ($G_{\alpha s}$) and stimulate AC activity and cAMP, while suppressing inflammatory cytokines (Rundhaug, Simper *et al.* 2011). The binding of PGE2 to either of these receptors would therefore lead to relaxation and suppression of inflammation (Slater, Astle et al. 2006) Figure 1.2). As such PGE2 may also have a role in maintaining quiescence (Figure 1.2).

Similar to PGE2 its receptors $EP_{1\text{-}4}$ are also regulated in a dynamic fashion during gestation and parturition (Olson 2003). The pro-contractile EP_3 receptor is down-regulated by 40% between non-pregnant and pregnant human myometrium (Matsumoto, Sagawa *et al.* 1997). In a primate model parturition was associated with a decrease in the pro-relaxatory EP_2 receptor (Olson 2003), and in a sheep model parturition was associated with an increase in the pro-contractile EP_3 receptor (Wu, Ma *et al.* 1999). Potentially therefore, tightly regulated temporal expression of specific prostaglandins and their associated receptors could regulate the contractility of the myometrium and hence the timing of labour.

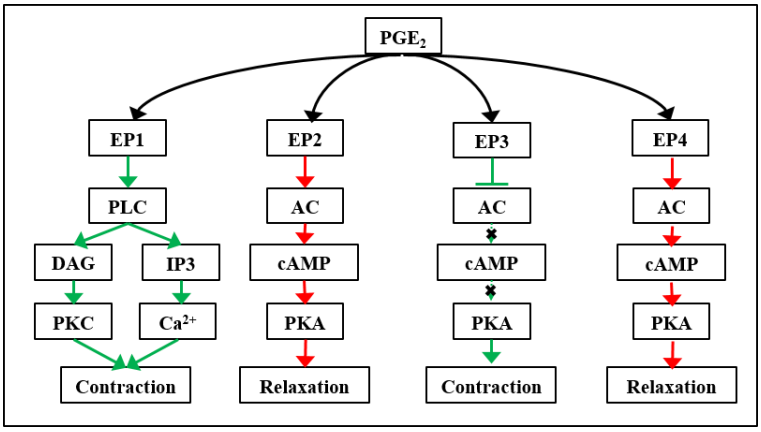


Figure 1.2 Signalling Pathways Activated by the EP Receptors for PGE2
EP_1 activates phospholipase C (PLC), resulting in the production of both 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). DAG activates protein kinase C (PKC) whilst IP3 results in the mobilisation of calcium. EP_3 blocks the conversion of adenylate cyclase (AC), reducing adenosine-3',5'-cyclic monophosphate (cAMP) levels leading to a reduction in protein kinase A (PKA). EP_2 and EP_4 couple to $G_{\alpha s}$ and stimulate AC activity and cAMP (Adapted with permission from (Rundhaug, Simper *et al.* 2011).

1.2.4 Transcriptional Regulation by NFκB

NFκB is involved in a variety of cellular processes including immune regulation, inflammation, cell cycle and apoptosis. NFκB is a rapid-acting transcription factor, a transcription factor that is present in the cell in an inactive state and so does not need new protein synthesis to be activated, meaning that it can be rapidly induced and acts as a first responder to cell stimuli (Perkins 2012).

NFκB has long been associated with inflammation, and is known to be activated by many of the cytokines which are prevalent during parturition such as TNF and IL-1β (Perkins 2007, Perkins 2012). In addition to this, many parturition associated genes are regulated by NFκB. A recent array study highlighted that NFκB influenced a wide variety of gene networks within the myometrium and many of these NFκB regulated genes have been shown to be differentially regulated during parturition (Chan, van den Berg *et al.* 2014).

NFκB is comprised of five subunits: p50; p52; p65 (RelA); cRel and RelB. Active NFκB consists of homo or heterodimers formed from the various subunits, the most prevalent of these being the p50/RelA heterodimer. Chapman *et al.* (Chapman, Europe-Finner *et al.* 2004) showed that there was a specific pattern of expression of each of the NFκB subunits in myometrium throughout human pregnancy and parturition. RelA, c-Rel, p100, p105 and p50 were all detected at high levels in the non-pregnant myometrium, however, there was a significant reduction of all of these, with the exception of RelA, in both pregnant

non-labouring and labouring myometrium. RelA was significantly reduced between pregnant and labouring myometrium, however the level of p50/RelA DNA binding was shown to increase. This increase in DNA binding was demonstrated using Electrophoretic Mobility Shift Assay (EMSA) and so the caveat to this is that it does not demonstrate binding in the context of the native promoter state (Saccani, Pantano *et al.* 2003), it also cannot provide any information on whether this regulation is stimulatory or inhibitory.

As stated previously, parturition is characterised by an increase in pro-inflammatory cytokines such as IL-1β, IL-6 and TNF (Romero, Mazor *et al.* 1992, Opsjln, Wathen *et al.* 1993, Keelan, Marvin *et al.* 1999, Osman, Young *et al.* 2003). This influx leads to the activation of NFκB, which in turn promotes the expression of pro-contractile genes such as PGF2α, Cox -2, oxytocin receptor (OR) and GAP junction protein Connexin 43 (Mendelson 2009; Figure 1.1). Studies in the mouse have shown that nuclear localisation of the p50 and RelA subunits increased closer to term and that administration of SN50, an NFκB inhibitor, delayed the onset of labour (Condon, Jeyasuria *et al.* 2004, Lindstrom and Bennett 2005). Unfortunately this study did not specifically determine that this increase in nuclear localisation was associated with an increase in DNA binding. Also SN50 is not a NFκB specific inhibitor it is in fact a more general inhibitor of nuclear importation (Boothby 2001). Therefore the delay in labour onset seen could be attributed to a general inhibition of the nuclear importation of transcription factors or co-factors.

A more recent study utilised ChIP-on-chip combined with expression array analysis to reveal the role of RelA-containing NFκB dimers in the regulation of parturition. The immortalised PHM1-31 human mymoterial cell line was utilised in this study with TNF stimulation used to represent the labouring state. The ChIP-on-chip analysis revealed that 13,300 genomic regions were bound by RelA-containing dimers in the presence of TNF and 11,110 genomic regions were bound in the un-stimulated cells. A portion of these bound regions were localised to genes known to be involved in parturition such as Cox-2 and Potassium Large Conductance Calcium Activated channel subfamily M Beta member 3 (KCNMB3). When this ChIP-on-chip data was combined with matched expression array analysis it revealed that 14 regions bound by RelA-containing dimers in the presence of TNF were up-regulated and a further five were repressed (Cookson, Waite *et al.* 2015). These genes with enriched RelA binding and up-regulated expression levels are also documented by Chan *et al.* (Chan, van den Berg *et al.* 2014) in their RNA-seq

study identifying differentially expressed genes in human myometrium from pregnant, non-labouring and actively labouring myometrium. Although this demonstrates that NFκB does have role in regulating gene expression during parturition the specifics of this role still need to be fully explored.

1.2.5 Progesterone Withdrawal

Evidence demonstrates that throughout pregnancy, progesterone acting via its nuclear receptor, regulates target genes to maintain uterine quiescence. At the end of pregnancy, in the majority of mammalian species, the levels of progesterone drops significantly and the uterus moves from a state of quiescence into a contractile state (Hardy, Janowski *et al.* 2006). In contrast to this, in humans, there is no reduction in circulating progesterone. Instead both progesterone levels and progesterone receptor levels remain elevated throughout pregnancy and into labour (Challis JRG, Matthews *et al.* 2000, Hardy, Janowski *et al.* 2006). It has been suggested, therefore, that a functional withdrawal of progesterone occurs through action on the progesterone receptors. There are three progesterone receptor isoforms: PR-A, PR-B and PR-C. PR-A and PR-B once stimulated, bind to progesterone receptor elements in the genome and regulate transcription. PR-C lacks the DNA binding domain and once bound sequesters progesterone away from the other receptors (Condon, Hardy *et al.* 2006). Studies utilising transfection techniques on various cell lines (MCF-10, HeLa, CV-1, MCF7) have demonstrated that these three isoforms have different effects on the genes they regulate. These effects were found to be strongly influenced by the cell and promoter context within which the receptor was acting and also which co-factors were recruited (Vegeto, Shahbaz *et al.* 1993, Chalbos and Galtier 1994, Wen, Xu *et al.* 1994). In most cell contexts PR-B enhances transcription while PR-A represses transcription and PR-C binds progesterone sequestering it away from PR-B (Vegeto, Shahbaz *et al.* 1993, Giangrande, Kimbrel *et al.* 2000, Condon, Hardy *et al.* 2006).

Studies in the PHM1-31 human myometrial cell line have shown that PGF2α increases the expression of PR-A mRNA but not PR-B mRNA. PGF2α has been shown to increase at the onset of parturition (Romero, Munoz *et al.* 1996, Brown, Alvi *et al.* 1998) which has led to the hypothesis that functional progesterone withdrawal occurs via the action of PGF2α promoting the increased expression of PR-A which in turn represses the

transcription of the pro-quiescent genes (Madsen, Zakar *et al.* 2004). This research, however, did not examine the effect of increased expression of PR-A on either gene expression or cell contractility. As regulation by PR-A and PR-B has been shown to be cell type and promoter context specific it would be necessary to look at the effect on gene expression within the PHM1-31 cells in order to prove or disprove this hypothesis.

When comparing PR expression between non-labouring and labouring human and mouse myometrium, Condon *et al.* noted there was a significant up-regulation of both the PR-B and PR-C isoforms in labouring tissue. Within the mouse, PR-B mRNA significantly increased with parturition in both the upper and lower myometrium, however, PR-B protein levels were only seen to increase in upper myometrium. There was also a 200-fold increase in the expression of the PR-C isoform, which they hypothesised could then act as an inhibitor of PR-B function and so may contribute to the functional withdrawal of progesterone (Condon, Hardy *et al.* 2006). However, PR are rapidly downregulated when cells are removed from the body and so this may affect the levels measured above.

1.2.6 Ion Channel Expression

Once the processes of fetal maturation, membrane rupture and cervical ripening and dilation are underway, the next step in the process is co–ordinated uterine contractions. The myometrial quiescent state is characterised by slow wave potentials where the membrane potential cycles between depolarisations and repolarisations without reaching the threshold level. However, within term pregnant myometrium, these slow wave potentials become frequent and synchronised action potentials during which the membrane potential rapidly rises and falls, causing the muscle to contract (Wilde and Marshall 1988).

Both the quiescent state and these co-ordinated contractions are thought to be mediated by ion channels, in particular the balance between the large conductance calcium activated potassium channel (KCNMA1, MaxiK, BK or *slo*-1) and the L-type calcium channel (CACNA1C).

1.2.6.1 The MaxiK Channel (KCNMA1)

It has been demonstrated that KCNMA1 plays a crucial role in regulating the contractility of the myometrium. Studies within the rat myometrium have revealed that KCNMA1 is differentially expressed throughout pregnancy. Non-pregnant and early pregnant rat myometrium express high levels of KCNMA1 protein and these levels are significantly reduced at the end of pregnancy and post-partum (Song, Zhu *et al.* 1999). KCNMA1 provides a strong repolarising current which would support myometrial quiescence. Therefore, the high levels of KCNMA1 protein seen early in pregnancy would help maintain the quiescent phenotype then, as the levels of KCNMA1 reduce towards parturition, the myometrium would switch to a more contractile state. This theory would need to be studied in human tissue in order to determine if it is relevant to human parturition.

1.2.6.2 L-Type Calcium Channel (CACNA1C)

The CACNA1C is thought to be a critical component of excitation-contraction coupling in smooth muscle and as such could contribute to the regulation of labour contractions. Studies within the rat myometrium revealed an increase in the number of CACNA1Cs within the membrane in late gestation. However, this work utilised the binding capacity of the CACNA1C inhibitor Dihydropyridine (DHP) as a measure of the number of channels and so this will not necessarily be a representative measure of active or functional channels. Although the number of CACNA1Cs was seen to increase there was no sharp increase immediately before or during parturition which seems to refute a role for these channels in triggering the onset of parturition (Mershon, Mikala *et al.* 1994). It is possible however, that changes in CACNA1C conductance rather than protein number may provide the trigger for parturition.

In these final stages of parturition there are clear indications of ion channel involvement. More research needs to be done to fully define the channels within the myometrium and how they work co-ordinately with each other to change the contractility of the tissue.

1.2.7 Summary of Current Theories

Although a considerable amount of research has been done to try and understand the processes which trigger the onset of parturition there is still no consensus on which

process or combination or processes are ultimately responsible for the switch to the labouring state. As such there is still a lack of understanding of the key steps involved in the switch from quiescence to contractility and further research into this is still needed.

1.3 Preterm Birth

Why is this research necessary? The human species is clearly surviving and expanding. The problem is that the process of parturition can and does go wrong, and when this happens it can result in preterm birth.

Each year preterm birth is the biggest cause of death in new born babies. In England and Wales nearly 8% (1 in 13) of live births are born prematurely (prior to 37 completed weeks of gestation). Evidence suggests that women who have had a previous preterm birth are two-and-a-half times more likely to have a further preterm birth than a woman who has not (Mercer, Goldenberg *et al.* 1999). Historically, research has focused primarily on improving the survival and health of preterm babies. Such efforts, however, have done nothing to decrease the incidence of preterm birth and in fact the incidence of severely preterm birth, which is prior to 26 completed weeks of gestation, is increasing (Goldenberg, Culhane *et al.* 2008, Chang, Larson *et al.* 2013).

Preterm birth can occur as a result of preterm premature rupture of membranes, labour induction or Caesarean delivery for maternal or fetal indication or following spontaneous labour. Current research suggests that iatrogenic causes of preterm labour include, infection, pre-eclampsia and intrauterine growth restriction. However, spontaneous labour accounts for 40-45% of preterm births and the cause of this is still poorly understood (Goldenberg, Culhane *et al.* 2008).

1.3.1 Current Tocolytic Therapies

Tocolysis, (Greek: *tokos* – childbirth; *lysis* – loosening, dissolving, dissolution) the inhibition of myometrial contractions, is currently the primary treatment for preterm birth, with the goal being to delay birth by a minimum of 18 hours. There are a number of tocolytics (anti-contraction medications) currently used which have been designed around our current understanding of the events leading up to parturition, which have been

described above. These include beta-adrenergic receptor agonists, calcium channel antagonists, oxytocin receptor antagonists and progesterone. These current tocolytics, however, are associated with contraindications for both mother and child (Gyetvai, Hannah *et al.* 1999).

1.3.1.1 β_2 Agonists

The β_2 Adrenergic Receptor (β_2AR) is associated with both $G_{\alpha s}$ and CACNA1C. Once activated $G_{\alpha s}$ activates AC by catalysing the formation of cAMP, which in turn activates PKA. PKA in turn acts on a number of smooth muscle structures such as; the sarcolemma, resulting in a decrease in calcium influx; the sarcoplasmic reticulum (SR), enhancing calcium uptake and the actin-myosin filaments, decreasing their interaction (1992) which ultimately leads to smooth muscle relaxation.

β_2 agonists activate this pathway and promote an increase in intra-cellular cAMP, a decrease in intracellular calcium, via increased SR uptake and decreased calcium influx (Figure 1.3; Anotayanonth, Subhedar et al. 2004). Within airway smooth muscle cells, treatment with β_2 agonists has been shown to cause KCNMA1 channels to open, leading to hyperpolarisation of the cells. The combination of decreased intracellular calcium, increased potassium efflux and decreased interaction of actin and myosin filaments leads to muscle relaxation.

There are three β2 agonists currently used: Terbutaline (currently used Off-Label as the FDA have not approved it for tocolysis), Ritrodrine and Salbutamol. These three are effective at delaying delivery by 24 to 48 hours with no significant differences seen between the effectiveness of each one. Treatment with these tocolytics beyond 48 hours has no effect on the reduction of preterm birth nor does it improve perinatal morbidity or mortality (Haram, Mortensen *et al.* 2003). Prolonged use of these drugs is associated with the phenomenon of receptor internalisation which results in desensitization of the target tissue to their relaxant effects. (van Geijn, Lenglet *et al.* 2005)

These β_2 agonists are not uterine specific and so all three are associated with side effects ranging from tremor, anxiety, palpitations, nausea and headache, and the more rare but serious side effects of pulmonary oedema (fluid accumulation in the lungs), myocardial

ischemia (restricted blood flow causing lack of oxygen to the heart) and cardiac arrest. They can also cause metabolic side effects such as hyperglycaemia, due to the non-specific activation of adrenergic receptors in the liver, leading to hyperinsulinemia (excessive circulating insulin) and finally to hypokalemia (excessive potassium in the blood) due to a shift in potassium caused by insulin. They can also pass through the placenta and may result in fetal tachycardia and fetal hypo- or hyperglycemia at birth (Chan, Cabrol *et al.* 2006, Wisanskoonwong, Fahy *et al.* 2011).

1.3.1.2 Calcium Channel Antagonists

Calcium Channel antagonists act by preventing calcium influx through membrane-spanning calcium channels. This leads to a reduction in intracellular calcium which, in turn, leads to a reduction in the creation of calcium:calmodulin complexes, a decrease in myosin light chain kinase (MLCK) phosphorylation and subsequently a decrease in myosin light chain phosphorylation. This then results in decreased myosin and actin interaction promoting smooth muscle relaxation (Figure 1.3).

There are two calcium channel anatgonists currently used: Nifedipine, which is used Off-Label, is not recommended for use with patients with hypertension as it can cause an increased risk of adverse cardiovascular events (Jaju and Dhabadi 2011), and Nicardipine (Laas, Deis *et al.* 2012). These have been shown to be capable of delaying birth up to 7 days, however, this has only been demonstrated with a total of 242 patients in 2 studies (van Geijn, Lenglet *et al.* 2005).

There are again side effects with the use of calcium channel antagonists but these are less severe than those seen with the β_2 agonists (Economy and Abuhamad 2001, King, Flenady *et al.* 2002, Haram, Mortensen *et al.* 2003). Nifedipine was also responsible for less severe side effects in comparison to Nicardipine (Laas, Deis *et al.* 2012). These side effects include dizziness, nausea, headache, weakness and oedema (fluid retention) and more rarely transient hypotension (abnormally low blood pressure; Dustan 1989, van Geijn, Lenglet et al. 2005). Like β_2 agonists, calcium channel antagonists can cross the placenta but data of the effect on the fetus is scarce.

A 2011 study demonstrated there was an increased risk of seizures among infants whose mothers were treated with calcium channel blockers, possibly due to the effect of decreased intracellular calcium in these infants. Neonatal seizures can be serious and can be associated with poor neurodevelopment (Davis, Eastman *et al.* 2011).

Unlike the β_2 agonists there is no reduction in efficiency with prolonged use of calcium channel antagonists (Economy and Abuhamad 2001, van Geijn, Lenglet *et al.* 2005).

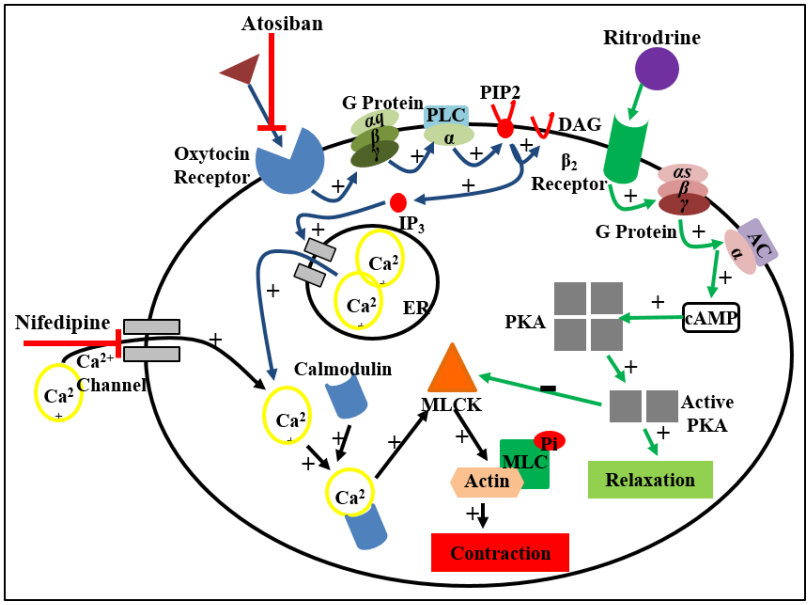


Figure 1.3 Mechanisms of Action for Tocolytics
Nifedipine, a calcium channel antagonist, promotes relaxation by blocking calcium influx through membrane-bound calcium channels and in doing so blocks this contractile pathway (black arrows). Atosiban, an oxytocin receptor antagonist, inhibits activation of the oxytocin receptor and the associated contractile pathway (blue arrows). Ritrodrine, a β_2 agonist, activates the β_2 adrenergic receptor and the associated pathway (green arrows) promoting relaxation.

1.3.1.3 Oxytocin Receptor Antagonist

The Oxytocin receptor is associated with $G_{\alpha q}$. Once activated, $G_{\alpha q}$ stimulates PLC which then generates both DAG and IP_3 through the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2). IP_3 is then released and binds to calcium channels located on the endoplasmic reticulum (ER); in turn this causes an increase in intracellular calcium and ultimately smooth muscle contraction (Gimpl and Fahrenholz 2001; Figure 1.3). Oxytocin receptor antagonists block activation of the oxytocin receptor and so block this signalling pathway thereby promoting smooth muscle relaxation.

There are currently two oxytocin receptor antagonist used for tocolysis: Atosiban and Barusiban (Reinheimer, Bee *et al.* 2005). Atosiban has been shown to be effective at delaying birth for up to seven days but had no significant effect in reducing the number of births prior to 37 weeks (Saez, Germain *et al.* 2003, Papatsonis, Flenady *et al.* 2005)

Again, oxytocin receptor antagonists can cause side effects including: headache, vomiting and nausea and, more rarely, tachycardia, hypotension and hyperglycaemia although less so than Ritodrine (Wex, Abou-Setta *et al.* 2011). Once again Atosiban crosses the placenta and, although it does not appear to accumulate in the foetus (de Heus, Mulder *et al.* 2010), it can cause fetal tachycardia (Wex, Abou-Setta *et al.* 2011).

1.3.1.4 Progesterone

The drawback of employing progesterone as a tocolytic is the fact that the mechanism of progesterone action is unclear. Progesterone is thought to modify the cellular structure of the myometrium through the inhibition of gap junction formation preventing co-ordinated muscular contraction (Garfield, Puri *et al.* 1982). It also blocks the action of oxytocin (Borna and Sahabi 2008).

Progesterone was shown to be effective in reducing pre-term birth prior to 37 weeks (Su, Samuel *et al.* 2010). Noted side effects of this drug, however, include headaches, nausea, breast tenderness and coughing. The long term potential for harm from the treatment is not known and research is needed in this (Wisanskoonwong, Fahy et al. 2011).

1.3.1.5 Summary

Tocolytics are only able to delay delivery temporarily and are rarely successful beyond 48 hours. This is sufficient time to allow the woman to be transferred to a specialist unit and for corticosteroids to be administered to reduce neonatal organ immaturity, but it is insufficient time for the baby to grow and fully mature (Gyetvai, Hannah *et al.* 1999, Chatterjee, Gullam *et al.* 2007).

An American study in 2003 demonstrated the cost burden of preterm birth. Briefly, the neonatal cost for a newborn weighing between 500-700g was $224,000 compared to $1,000 for a newborn weighing over 3000g. These costs increased exponentially as

gestational age and weight decreased (Gilbert, Nesbitt *et al.* 2003). This study did not take into consideration any longer term care.

Preterm birth, however, does not only affect the infants in the weeks and months following birth but in fact one-in-ten babies born severely preterm will develop a permanent disability such as lung disease, cerebral palsy, blindness or deafness (Marlow, Wolke *et al.* 2005). Taking into account these additional long-term costs a British study calculated that delaying birth by as little as a week would save the UK economy up to £260m a year (Mangham, Petrou *et al.* 2009).

Evidently it is vitally important to develop better strategies for preventing preterm birth both in terms of the health of the babies involved and the health care costs (2008), and this can only be done by understanding the processes which move the myometrium from its relaxed quiescent state to the contractile state seen at parturition. The development of such strategies must begin with understanding the fundamental nature of myometrial smooth muscle and its contractile mechanisms.

1.4 Smooth Muscle Structure

There are three varieties of muscle: smooth, cardiac and skeletal. These all contract by sliding thin actin filaments past stationary thick myosin filaments in response to an increase in calcium and they all use adenosine triphosphate (ATP) as the energy source for this process. This, however, is where the similarities end as each muscle type has a different; structure, organisation, mechanism of excitation, excitation-contraction coupling, and response to contraction.

Smooth muscle is mostly located within hollow organs and vessels including the vascular system, or the digestive tract where contraction acts to move the contents of the tube forward. In hollow organs, such as the bladder or uterus, contraction acts to expel the contents of the organ.

Smooth muscle cells are spindle shaped, with a single nucleus and are much smaller than the multi-nucleated skeletal muscle cells. Smooth muscle itself is further sub-categorised as either single unit or multi-unit smooth muscle. Multi-unit smooth muscle is rare and

can be found in the eye muscles (related to distance vision), the base of hair follicles and the iris. Within this type of muscle tissue the smooth muscle cells are organised into functional units which are then separately stimulated by the autonomic nervous system. Each of these units is functionally independent from the other units. Single-unit smooth muscle is more common; it can be found in the stomach walls and the uterus. Within this type of muscle tissue the smooth muscle cells are organised into sheets, with the cells lying roughly parallel to each other. Each of the cells is connected to its neighbour via gap junctions and they contract together as a single unit. Gap junctions act as low resistance pathways which enable electrical signals to rapidly spread throughout the tissue. This tissue is stimulated both through the autonomic nervous system and myogenic activity.

1.4.1 Molecular Basis of Smooth Muscle Contraction

When calcium channels on the smooth muscle membrane open and calcium diffuses into the cell, this increase in intracellular calcium causes the SR to release small quantities of calcium. Four calcium ions then bind to each calmodulin, a messenger protein which binds calcium ions and modifies their interactions with various proteins. In this instance activated calmodulin activates MLCK, which in turn phosphorylates myosin through the conversion of ATP to adenosine diphosphate (ADP). Finally, phosphorylated myosin binds with actin and cross bridge cycling begins (Figure 1.4).

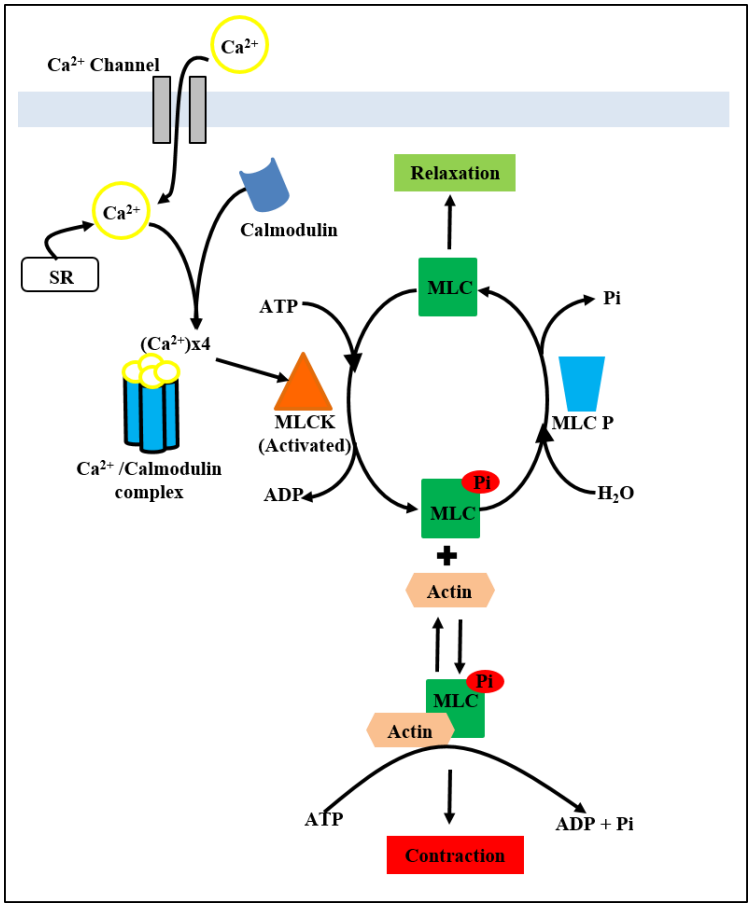


Figure 1.4 Molecular Mechanism of Contraction
Schematic of the molecular basis of smooth muscle contraction. Calcium enters the cell via calcium channels and causes the release of calcium from the sarcoplasmic reticulum (SR). This calcium then binds to calmodulin forming a complex which then activates myosin light chain kinase (MLCK). Once activated MLCK then phosphorylates the myosin light chain (MLC) which then binds to actin causing contraction. Myosin Light Chain Phosphatase (MLCP) removes the phosphate group from MLC and leads to relaxation.

The cross bridge cycle is the process by which the chemical energy stored in the terminal phosphate group of ATP is converted into movement. It begins with the binding of phosphorylated myosin to actin, followed by the release of the phosphate group which causes a conformational change in myosin, which leads to the power stroke, causing the filaments to slide and ADP to be released. A new ATP binds to myosin, allowing it to be released from actin. The ATP is converted to ADP and myosin returns to its original conformation. The cycle then begins again (Figure 1.5).

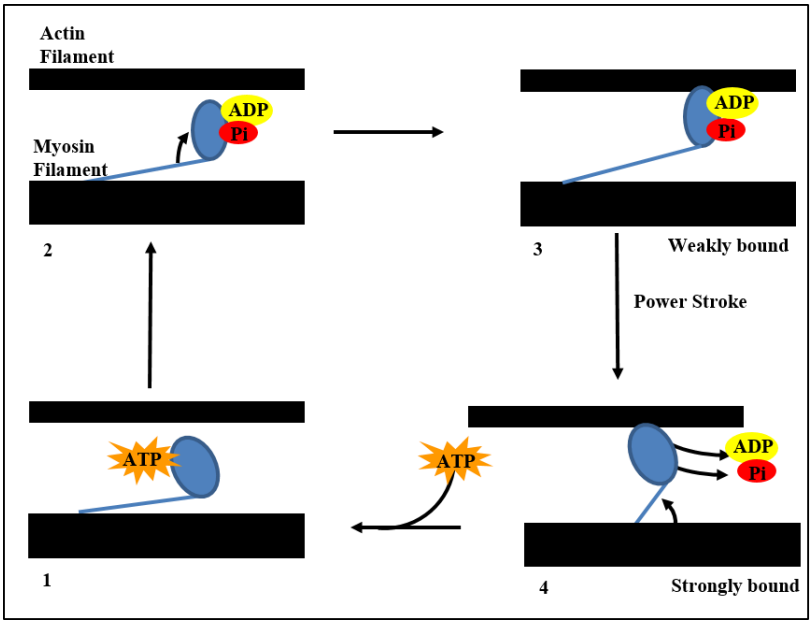


Figure 1.5 Cross Bridge Cycling
Schematic representation of the Cross Bridge cycling process. 1) ATP binds to the myosin head in its low energy configuration; 2) ATP is split into ADP and Pi and the myosin head cocks; 3) The myosin cross bridge attaches to the actin filament with the myosin head in the high energy configuration; 4) During the power stroke ADP and Pi are released from the myosin head and the head pivots and bends as it pulls the actin filament.

Relaxation occurs when calcium is removed from the smooth muscle cell via the use of the sodium calcium exchanger or the plasma membrane calcium ATPase transporter protein (PMCA) or the Sarco/Endoplasmic reticulum calcium ATPase transporter protein (SERCA) or a combination of these. The phosphate group is then removed from myosin by myosin light chain phosphatase (MLCP), meaning that myosin can no longer bind to actin to form the cross bridges.

1.4.2. Stimulation of Smooth Muscle Contraction

Smooth muscle can be stimulated electrically. Single-unit smooth muscles, such as lymph vessels, have pacemaker regions. In these regions contractions are spontaneously and rhythmically generated because of automatic changes in cell permeability. Once an action potential is fired in a pacemaker cell, it will then spread to the rest of the smooth muscle cells via gap junctions. In the intestine the membrane potential gradual alternates between hyperpolarizing and depolarizing potentials. This is termed slow wave potential and is caused by cyclical changes in the active transport of sodium. Although this does not always result in an action potential, when it does it again spreads across the whole tissue.

A second excitation mechanism in smooth muscle is based on receptor activity; this form of stimulation is independent of changes in membrane potential. Increases in IP3, through activation of $G_{\alpha q}$ and PLC, causes a release of calcium from intracellular stores and then, via PKC, cause an influx of calcium through the CACNA1C. The activity of MLCP can also be inhibited through antagonists acting through a G protein. This reduction in MLCP activity results in increased myosin phosphorylation and hence increased contraction (Christopher 1996).

Finally, smooth muscle can be stimulated by mechanical activity i.e. stretching. Stretching either arterial or uterine muscles induces light chain phosphorylation to the same extent as muscles contracted by either potassium or norepinephrine. Once the stretch is released the contraction which follows is spontaneous. However, if either a chelating agent such as ethylene glycol tetraacetic acid (EGTA), or a calmodulin inhibitor such as chlorpromazine, is added, both the light chain phosphorylation and upon release of the stretch the spontaneous contraction is abolished. This indicates that mobilisation of calcium is needed for both these activities. It is possible that these effects are the result of mechanosensitive receptors in the smooth muscle interacting with calcium release channels.

1.4.3 Myometrial Contractility

The myometrium is composed of smooth muscle cells embedded in an extracellular matrix of collagen fibres and functions as single-unit smooth muscle. In the same way as single-unit smooth muscle, the myometrial muscle cells communicate with each other via gap junctions. The contractile state of the myometrium is predominantly controlled by intracellular calcium ion concentration.

1.4.3.1 Resting Membrane Potential

The resting membrane potential of myometrial myocytes has been estimated to be between -35 and -80V (Kao 1989, Parkington and Coleman 1990, Sanborn 2000). Similar to the intestine this resting potential gradually alternates between hyperpolarizing and depolarizing potentials, i.e. slow wave potential. The oscillations in membrane potential correlate with the distribution of calcium, sodium, potassium and chloride ions and the permeability of the cell membrane. Within the myometrium, the cellular excitability is

dependent on the movement of sodium, chloride and calcium ions into the cells and potassium ions out of the cells. Calcium is the key ion for increasing cellular excitability. This arises as calcium ions have the largest electrochemical gradient so there is a rapid and significant rise in calcium ion concentration upon the opening of calcium channels in the membrane, whereas resting potential is maintained by potassium channels. Potassium channels conduct an outward current while the muscle is inactive to maintain the resting potential. Also, after stimulus this outward current conducted by the potassium channels repolarises the membrane and hence decreases the excitability of the cell (Figure 1.6). The KCNMA1 channel is thought to be the key potassium channel to conduct these currents within the myometrium as it is able to respond to changes in calcium as well as membrane potential (Aguilar and Mitchell 2010).

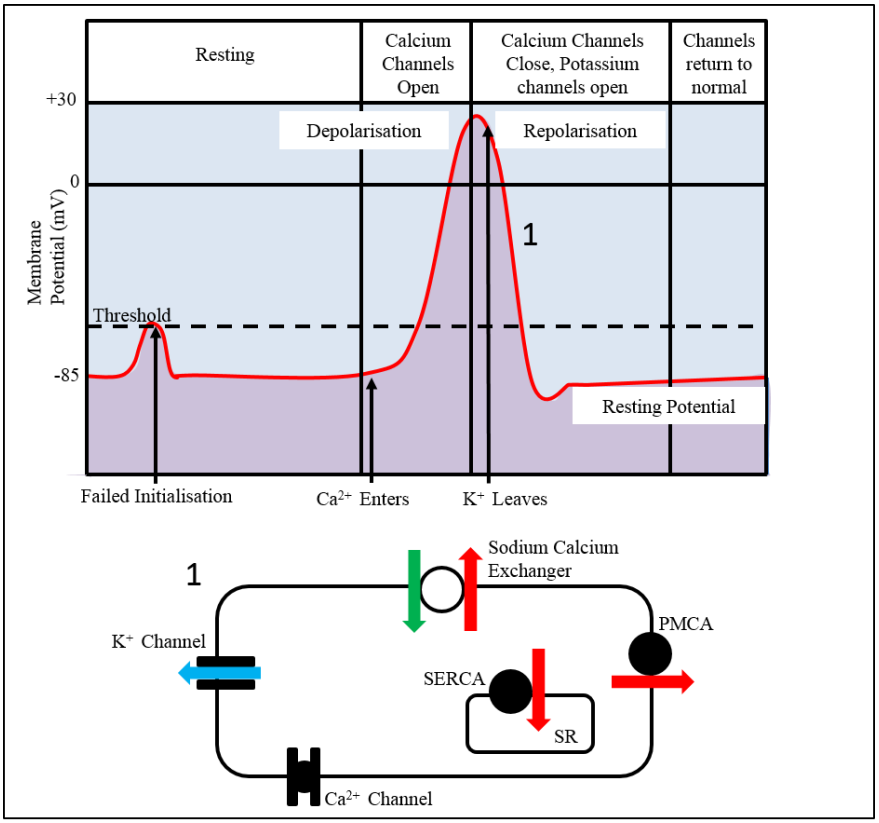


Figure 1.6 Schematic Representation of the Generation of an Action Potential
Briefly, the membrane potential gradually rises and falls due to ion flux across the membrane but these fluctuations do not cross the threshold level. Then, once the calcium channels open, calcium floods into the cell and the cell membrane rises (depolarisation) and an action potential is generated. Subsequently, when potassium channels open, potassium leaves the cell, the cell membrane potential falls (repolarisation), the calcium channels close and the action potential is terminated. Calcium is also removed from the cytoplasma by the combined action of the plasma membrane calcium ATPase (PMCA), sarco/endoplasmic menmbrane calcium ATPase (SERCA) and the sodium calcium exchanger, the cell then returns to its resting state.

1.4.3.2 Generation of Action Potentials

Contractile activity within the myometrium, like other smooth muscles, is mediated by changes in intracellular calcium ion concentration. The CACNA1C is the predominant channel within the myometrium (Tezuka, Ali *et al.* 1995, Parkington, Tonta *et al.* 1999, Collins, Moore *et al.* 2000). When the membrane depolarises to ~ -40mV, the CACNA1Cs open and calcium ions flood in causing a significant increase in intracellular calcium concentration. This increase in intracellular calcium activates the contractile pathway described above.

T-type calcium channels (CACNA1G) have also been found in the myometrium (Fry, Sui *et al.* 2006, Blanks, Zhao *et al.* 2007, Lee, Ahn *et al.* 2009). It is thought that these channels use their faster gating kinetics to propagate the action potential, or possibly by opening at more negative voltages they are able to elevate the membrane potential to the voltage necessary for the CACNA1C to open.

Once an action potential has been generated this is then transmitted to the rest of the tissue via gap junctions. Garfield *et al.* demonstrated that the number of gap junctions within the myometrium increased in the final stages of pregnancy up until the start of parturition (Garfield, Puri *et al.* 1982). This increase in gap junctions forms an electrical syncytium which is necessary for the generation of co-ordinated myometrial contractions.

1.4.3.3 Return to Resting Membrane Potential

Once the contraction has taken place it is necessary for the calcium to be removed, in order to promote relaxation of the muscle and to allow the SR to replenish its calcium stores in preparation for the next contraction.

Calcium can be removed from the cell in a number of ways. One such way is the use of the sodium calcium exchanger. In simple terms this exchanger utilises the energy from the electrochemical gradient of sodium, allowing sodium to flow down the gradient into the cell in exchange for the removal of calcium ions (Blaustein and Lederer 1999, Noble and Herchuelz 2007). Another method utilises the PMCA. This transporter protein functions by hydrolysing ATP to ADP, with one calcium ion being removed for each molecule of ATP hydrolysed (Noble and Herchuelz 2007). The SERCA functions in the

same way as the PMCA, with the exception that it transports the calcium from the cytosol into the lumen of the SR. In this way the SR can replenish its stores of calcium (Franklin, Winz *et al.* 2001, Borge, Moibi *et al.* 2002).

It has been demonstrated that the expression of PMCA and SERCA protein is increased in human myometrium during parturition. This would allow for a more rapid recovery after a contraction and so these may have a functional role in the regular contractions seen during parturition (Tribe, Moriarty *et al.* 2000).

1.4.4 Regulation of Uterine Contractility

The non-pregnant myometrium contracts as a reflex response to stretch. During pregnancy this reflex response is suppressed and the myometrium enters a state of relaxation termed quiescence. Prior to parturition the myometrium again becomes contractile initially, experiencing weak irregular contractions which culminate with a relatively short burst of strong rhythmic contractions at parturition.

Although the molecular basis of contraction is understood, the triggers which move the myometrium from the quiescent state seen during gestation to the contractile state seen at parturition are poorly understood.

It has been suggested that calcium sensitisation may play a role in this switch. Calcium sensitisation is the phenomenon by which a given concentration of intracellular calcium results in a larger than expected force of contraction (Ratz, Berg *et al.* 2005, Arthur, Taggart *et al.* 2007, Wray 2007). Inhibition of myosin light chain phosphatase has been suggested to be the mechanism through which calcium sensitization occurs (Uehata, Ishizaki *et al.* 1997). Another putative mechanism for calcium sensitization is via the loss or reduction in calcium sensitivity of calcium-activated potassium channels. A reduction in, for example, KCNMA1 channel calcium sensitivity would result in a reduction in the repolarising current supplied by these channels and hence an increase in contractility.

1.5 Calcium Activated Potassium Channels and Voltage-Dependant Calcium Channels Within the Myometrium

Calcium-activated potassium channels, such as the KCNMA1 channel, have an important role regulating action potentials in some smooth muscles. By responding to calcium influx they are able to maintain relaxation. Calcium channels such as the CACNA1C and CACNA1G, voltage-dependant calcium channels, regulate the flow of calcium into the cell and hence control the contractility of the muscle. It has been shown that calcium release from the SR causes the CACNA1Cs to open, which in turn causes myometrial contractions. The increased intra-cellular calcium level, however, then activates the KCNMA1 channel which in turn leads to relaxation (Figure 1.7; Chanrachakul 2006). In theory, the balance between these calcium and potassium channels could result in either quiescence or contractility within the myometrium.

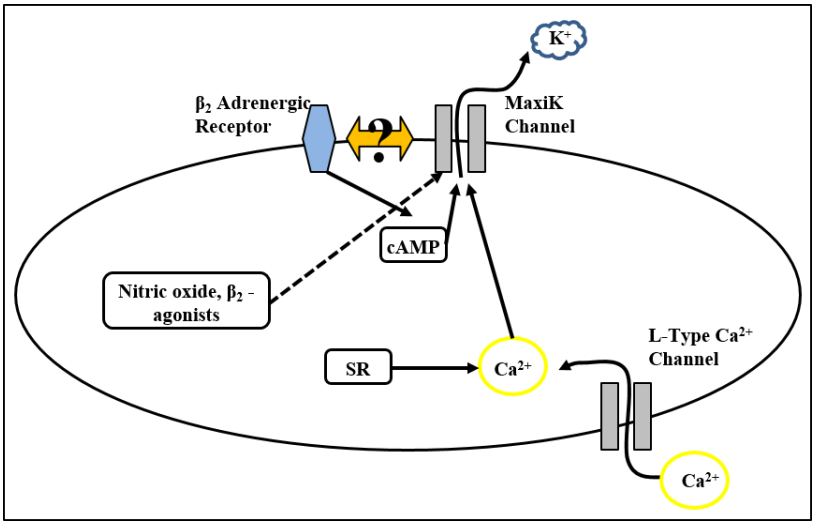


Figure 1.7 Contraction/relaxation Mechanisms
A simplified scheme of the contraction/relaxation mechanisms of pregnant human myometrial cell (Adapted with permission from Chanrachakul 2006).

1.5.1 The KCNMA1 (BK or *slo*-1) Channel

KCNMA1 channels are integral membrane proteins that contain seven membrane-spanning domains, a pore-forming domain and a cytoplasmic tail region containing four hydrophobic domains, and a calcium bowl. KCNMA1 channels can be formed by two subunits, the pore-forming α-subunit and the modulatory β-subunit (Figure 1.8).

Activation of the KCNMA1 channels results from changes in membrane electrical potential and/or increasing concentrations of intracellular calcium ions (Ca^{2+}; Miller

2000, Yuan, Leonetti et al. 2010), making it a unique member of the potassium channel family. The effect of this dual sensitivity is that the KCNMA1 channel can open at increasingly negative membrane potentials. Once open, potassium ions passively flow through the channel, down the cells electrochemical gradient. This leads to a reduction in intracellular potassium levels and hence membrane hyperpolarisation, resulting in decreased cell excitability. Intracellular calcium also regulates the physical association between the α- and β- subunits.

The link that the KCNMA1 channel provides between free calcium in the cytosol and membrane potential is thought to have an important role in membrane excitability (Cui, Cox *et al.* 1997, Schreiber and Salkoff 1997, Schreiber, Yuan *et al.* 1999). There is, however, a high degree of functional variability observed in the KCNMA1 channel and a large proportion of this is related to its calcium and voltage sensitivity (Lagrutta, Shen *et al.* 1994).

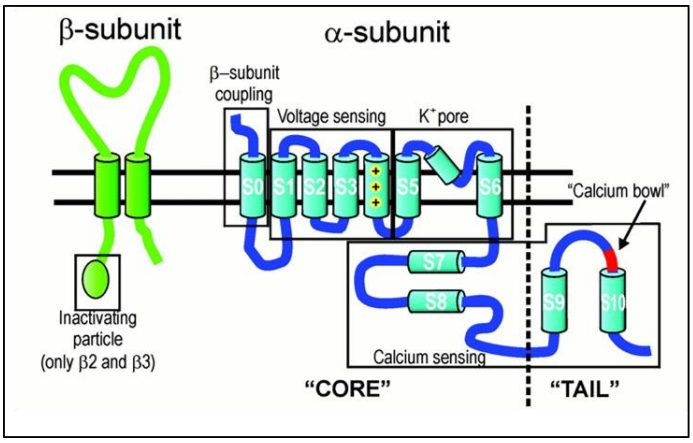


Figure 1.8 The KCNMA1 Channel
A schematic representation of the Maxi K channel. The α-subunit is comprised of an extracellular N-terminus followed by seven transmembrane-spanning domains (S0-S6), with the pore (p) located between S5 and S6, and a cytoplasmic 'tail' containing four hydrophobic domains and a calcium bowl and ends with an intracellular C-terminus. The β-subunit consists of and intracellular N-terminus followed by two trans-membrane spanning domains and then an intracellular C-terminus (Adapted with permission from Orio, Rojas et al. 2002)

1.5.1.1 Calcium Sensitivity in the KCNMA1 Channel

Calcium sensitivity is thought to be derived from an EF motif in the tail region of the KCNMA1 Channel (Korovkina and England 2002). An EF motif is comprised of two alpha-helices (E and F) joined by a loop; calcium is bound by that loop region. Braun and Sy used mutational analysis combined with transfection and electrophysiology to locate

putative EF motifs in the mouse brain *mSlo* α-subunit. Their findings indicate that there are two imperfect EF structures – one located in the N terminal region and one overlapping the calcium bowl in the C terminal region (Braun and Sy 2001).

The calcium bowl is a highly conserved segment of the KCNMA1 protein which is located between S9 and S10 (Schreiber and Salkoff 1997) and the remarkable level of conservation between species would indicate that this region is a functionally important part of the channel. The calcium bowl contains a number of aspartic acid residues which cause the region to carry an extremely negative charge. Aspartate is known to co-ordinate the calcium ions within calcium binding proteins. Mutations abolishing the calcium sensitivity of the calcium bowl results in a channel which opens more easily at physiological voltages with the effect of reducing membrane excitability (Schreiber, Yuan *et al.* 1999). This research indicates that the calcium sensing region of the KCNMA1 channel is predominately located in the C-terminal tail region.

1.5.1.2 Voltage Sensitivity in the KCNMA1 Channel

Cui *et al.* showed that the voltage sensitivity of the channel is intrinsic to the channel itself and independent of calcium binding (Cui, Cox *et al.* 1997). Voltage sensitivity of the KCNMA1 channel is thought to be found in the S1 – S4 domain. Within this, the S3 / S4 regions contain some conserved charged residues and a series of three regularly spaced arginine residues essential in voltage dependent gating (Meera, Wallner *et al.* 1997, Ma, Lou *et al.* 2006). Mutational analysis of the charged residues in regions S1 - S4, showed that although S4 contains five charged residues, only one of these actually contributes to the channel's gating charge. This is in sharp contrast to the Shaker channel in which five of the seven charged residues in S4 contribute to its gating charge (Ma, Lou *et al.* 2006). This would account for the lower voltage dependence of the KCNMA1 channel compared to the Shaker channel despite similarities in structure. Mutation of the charged residues in S2 and S3 showed that these residues accounted for ~50% of the voltage sensitivity of the KCNMA1 channel. For a review of the different potassium channels see Sansom *et al.* (Sansom, Shrivastava *et al.* 2002).

Koval *et al.* extended this mutagenesis-based study to include the S0 region, which is unique to the KCNMA1 channel. This region is highly conserved between different

species and so is thought to be functionally important either as a link between the α- and β-subunits or as part of the voltage sensing mechanism (Koval, Fan *et al.* 2007). Mutation of a number of conserved residues within the S0 region was shown to greatly alter the gating of the channel (Koval, Fan *et al.* 2007). The mechanism of this alteration would suggest a multifaceted role of the S0 region including the modulation of active and resting state equilibrium, the transduction of β-subunit actions and the stabilisation of the resting state channel (Liu, Li *et al.* 2000). The voltage sensitivity is therefore a property of the N-terminal trans-membrane segments of the KCNMA1 Channel.

1.5.1.3 Splice Variants in the KCNMA1 Channel

Splicing of the KCNMA1 channel transcript has been shown in species as diverse as *Drosophila* (Adelman, Shen *et al.* 1992, Lagrutta, Shen *et al.* 1994, Wei, Solaro *et al.* 1994); Mouse (Pallanck and Ganetzky 1994, Wei, Solaro *et al.* 1994, Benkusky, Fergus *et al.* 2000, Holdiman, Fergus *et al.* 2002); Chicken (Navaratnam, Bell *et al.* 1997); Bovine (Knaus, Eberhart *et al.* 1995) and Human (Dworetzky, Trojnacki *et al.* 1994, Pallanck and Ganetzky 1994, Tseng-Crank, Foster *et al.* 1994, Korovkina, Brainard *et al.* 2006, Davies, Zhao *et al.* 2007, Korovkina, Stamnes *et al.* 2009) this is not surprising considering the sequence homology found between these species (~92%). Alternate splicing of the KCNMA1 channel can alter its calcium sensitivity, voltage sensitivity, sensitivity to protein phosphorylation and cellular localisation. This provides a mechanism for fine tuning the channels response to a diverse range of regulatory and conductance requirements.

Splice variants can be generated by various means such as the alternative use of cassette exons or mutually exclusive exons, alternative splice donor sites, alternative splice acceptors sites and intron retention. A cassette exon is the name given to an optional exon which can be retained by optional splicing within an intron. When one of two exons is retained, but not both these are termed mutually exclusive exons. Splice donor sites cause either elongation or shortening of the preceding exon by splicing at an alternative intron sequence near the 5' end. Splice acceptor sites cause either elongation or shortening of the following exon by splicing at an alternative intron sequence near the 3' end (Figure 1.9; Jurkat-Rott and Lehmann-Horn 2004). There are a number of splice variants found in the human KCNMA1 channel, which are summarised in Table 1.1.

Korovkina *et al.* demonstrated the presence of a 132 base pair (bp) exon (M1 / Mk44; accession number AF349445) which, if inserted, would result in a 44 amino acid (aa) segment being inserted into the S0 – S1 linker region. This forms a functional channel with diminished sensitivity to intracellular calcium and also voltage. The amino acid sequence of the insert contains two consensus sites for protein phosphorylation and also a myristylation site (Korovkina, Fergus *et al.* 2001, Korovkina, Brainard *et al.* 2006).

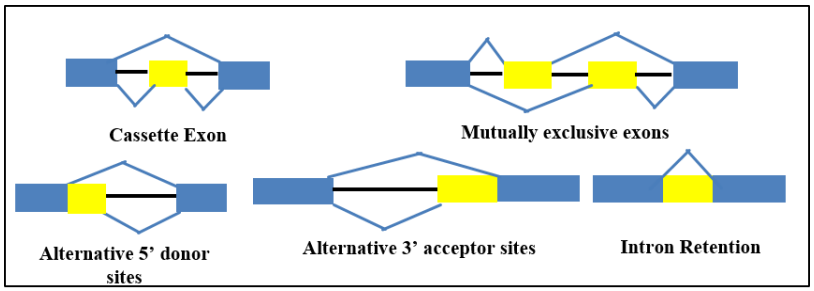


Figure 1.9 Splicing Events
Diagrammatic representation of the basic types of alternate splicing events (Gathman 2009)

Phosphorylation alters channel function (Lieberman and Mody 1999). Yan *et al.* demonstrated that phosphorylation of affinity purified KCNMA1 channels from rat brain membranes altered the channels dependence on voltage and calcium for activation (Yan, Olsen *et al.* 2008). Phosphorylation can also have a role in protein-protein interactions, cell membrane localisation and subcellular targeting.

Myristylation has an important function in membrane localisation of proteins and interaction between different subunit proteins (Raju, Kakkar *et al.* 1997). Given that membrane localisation of the KCNMA1 channel is essential for its activity and that association with its accessory β-subunit can affect calcium sensitivity, the presence of a myristylation site within this splice variant is worthy of further investigation.

Korovkina *et al.* went on to show that the Mk44 variant undergoes proteolytic digest. The N-terminus was localised to the cell membrane using co-localisation experiments. However, Korovkina demonstrated that the Mk44 C-terminus was not expressed on the cell membrane but instead was intracellular and localised specifically with the endoplasmic reticulum (Korovkina, Brainard *et al.* 2006). After calcium release from the

sarcoplasmic reticulum-based stores, however, the C-terminus was found to translocate to the membrane and reconstitute with the N-terminus, forming a functional channel.

Table 1.1 KCNMA1 Splice Variants
Splice variants found in the human KCNMA1 Channel, their location, exon profile and the effects they have on channel function.

Locus	Channel Region	Splice Variation Profile	Putative Effects	References
M1 Mk44	S0-S1 linker	132bp insert between exons 1 and 2	Decreased voltage and Ca^{2+} sensitivity	(Korovkina, Fergus *et al.* 2001, Curley, Morrison *et al.* 2004)
M2	S6-S7 linker	Mutually exclusive use of exons 10 and 11 or 11 and 12	Truncation of the protein	(Curley, Morrison *et al.* 2004)
M3 SRKR	S8-S9 linker	3 variants of exon 19 – a 3' truncation, skipping of exon 19 or the use of the entire exon	unknown	(Tseng-Crank, Foster *et al.* 1994, Curley, Morrison *et al.* 2004, Davies, Zhao *et al.* 2007)
M4 STREX	S8-S9 linker	Differential utilization of exons 22 and 23 (deletion of both – insertless, inclusion of 22 – STREX-1 and inclusion of both – STREX-2)	Mechano-sensitivity Hypoxia inhibition Altered response to PKA	(Curley, Morrison et al. 2004, Davies, Zhao et al. 2007, Lu, Alioua et al. 2006, Pietrzykowski, Friesen et al. 2008)
M4 IYF	S8-S9 linker	Inclusion of exon 22	Hypoxia insensitivity	(Zarei, Zhu *et al.* 2001, Davies, Zhao *et al.* 2007)
M5 Ca27	S9-S10 linker	Insertion or deletion of exon 29	Increased activation rate	(Tseng-Crank, Foster *et al.* 1994, Yan, Olsen *et al.* 2008)

Davies *et al.* investigated a splice variant in the S8 – S9 linker region. The identified two variants; SVcyt which contained an additional 255bp and SV0 which lacked the 255bp insert. They found that SVcyt was retained within the cytoplasm, while SV0 was expressed on the membrane (Davies, Zhao *et al.* 2007). SVcyt was found to be an inactive form of the KCNMA1 channel while SV0 was active.

The SVcyt splice variant is actually a collection of three distinct but adjacent splice sites which have been reported in the S8-S9 linker region. There is some difficultly correlating

the research of these different splice sites as there is no common nomenclature used for the different sites.

There is a 12bp insert known as SRKR (M3), within the S8-S9 region. However, the effect of this insert on human channel function has not been fully explored (Tseng-Crank, Foster *et al.* 1994).

The STress Regulated EXon (M4 / STREX), also located between S8 and S9, has been shown to increase the mechano-sensitivity of the channel (Lu, Alioua *et al.* 2006, Wang, Huang *et al.* 2010) and to confer hypoxia inhibition (McCartney, McClafferty *et al.* 2005). The STREX insert also contains a cAMP dependant PKA consensus motif. Phosphorylation of the KCNMA1 channel with the STREX insert by PKA facilitates membrane depolarisation. However, PKA phosphorylation of the KCNMA1 channel without the STREX insert facilitates membrane repolarisation. In simple terms phosphorylation by PKA in the presence of STREX facilitates contraction and phosphorylation in the absence of STREX facilitates quiescence (Tian, Coghill *et al.* 2004).

An alternative sequence to the STREX insert is the amino acid triplet IYF (M4; Appendix 1 *Amino acid code*), which confers insensitivity to hypoxia (McCartney, McClafferty *et al.* 2005). Also reported in this region is the Ca27 (M5) insert (Tseng-Crank, Foster *et al.* 1994, Yan, Olsen *et al.* 2008) which is found proximal to the calcium bowl and contains a phosphorylation site. Channels containing the Ca27 insert have increased activation rates and their co-operativity with calcium is modified (Ha, Jeong *et al.* 2000, Yan, Olsen *et al.* 2008), possibly due to its proximity to the putative EF hand motif and calcium bowl.

The differential expression of KCNMA1 isoforms in different tissues and species, and the fact that multiple isoforms can be present in a single cell, combined with different research techniques – whether RNA transcription or protein expression was studied or if whole cell protein or membrane protein was extracted and the tissue type used - means that it is difficult to combine this extensive research into one complete picture.

1.5.1.4 KCNMA1 Channel Expression During Gestation

The expression of different KCNMA1 channel isoforms is both tissue- and stimulus-specific. During gestation, the myometrial cell is increasingly permeable to calcium and membrane potential increases to more depolarised potentials at term (Benkusky, Fergus *et al.* 2000). Therefore, alternative splicing of the KCNMA1 channel may be a mechanism by which uterine contractility can be modulated during gestation.

The non-pregnant myometrium naturally contracts when subjected to stretch but the pregnant myometrium must stretch to accommodate the growing foetus. This reflex contraction must be inhibited until the initiation of labour, when the myometrium must rhythmically contract to expel the fetus. Calcium channels are responsible for the contractility of smooth muscle, and the calcium-activated potassium channels counteract the influx of calcium and maintain smooth muscle relaxation. A fine balance of these two channels would appear to be essential in the maintenance of quiescence during gestation and then the progression into labour.

There is general agreement that the predominant potassium channels in myometrial cells are KCNMA1 channels (Khan, Smith *et al.* 1993, Song, Zhu *et al.* 1999, Curley, Morrison *et al.* 2004) and that the calcium sensitivity of these channels decreases at the onset of labour (Khan, Smith *et al.* 1993, Khan, Smith *et al.* 1997, Song, Zhu *et al.* 1999, Curley, Morrison *et al.* 2004). There is much less agreement, however, on whether this reduction in calcium sensitivity is due to reduced expression of the channel (Chanrachakul, Matharoo-Ball *et al.* 2003, Gao, Cong *et al.* 2009) or if it is due to a change in splice variant expression (Khan, Smith *et al.* 1997).

Gao *et al.* demonstrated that the expression of the α- and β-subunits is significantly decreased between pregnant and labouring samples in both the upper and lower segment myometrium, but the portion of the various splice variants present were not examined (Gao, Cong *et al.* 2009). Other research has reported a switch in the splice variant expression between non-labouring and labouring myometrium, but these studies tend not to put this in the context of overall expression of the KCNMA1 channel (Curley, Morrison *et al.* 2004).

The Mk44 splice variant has been shown to be present in the human myometrium (Curley, Morrison *et al.* 2004) and has been shown to be up-regulated in labouring myometrium (Curley, Morrison *et al.* 2004). This particular splice variant has decreased calcium and voltage sensitivity and so could explain the decreased calcium sensitivity of KCNMA1 channels found in labouring myometrium.

The expression of a second splice variant, Ca27, was found to be unchanged in labouring myometrium. Indeed, this particular variant is responsible for increased activation of the KCNMA1 channel. Faster activation of the KCNMA1 channel would result in a faster response to membrane depolarisation. This variant may then contribute to the maintenance of quiescence.

Finally, although STREX variant expression in the myometrium has not been directly studied, Zhu *et al.* have looked at the effect of PKA in pregnant and non-pregnant myometrium (Zhu, Eghbali *et al.* 2005). cAMP and cyclic guanidine monophosphate (cGMP) can activate potassium channels and specifically the KCNMA1 channel and in this way may contribute to the maintenance of quiescence. KCNMA1 channels phosphorylated by PKA via cAMP, and by endogenous membrane bound Protein Kinase G (PKG) via cGMP generate increased activity in the pregnant myometrium (Zhou, Wang *et al.* 2000). This increased activity would inhibit the natural contractile response to stretch. Conversely in non-pregnant myometrium, PKG has been shown to inhibit KCNMA1 channels. The switch between enhancing and inhibitory effects of PKA / PKG between pregnant and non-pregnant myometrium would indicate that there are different splice variants present whose response to PKA / PKG differs. This switch effect correlates well with the known properties of the STREX variant, specifically the facilitation of membrane depolarisation following PKA phosphorylation when STREX is present, and facilitation of membrane repolarisation following PKA phosphorylation when STREX is absent.

The activation of PKA is dependent on the presence of cAMP. Essentially, cAMP being formed as a result of AC-mediated catalytic cyclization of ATP. This process is dependent on the $G_{\alpha s}$ subunit of a stimulated G-protein complex. Europe-Finner *et al.* demonstrated that $G_{\alpha s}$ levels in the myometrium were increased during pregnancy and decreased during labour (Europe-Finner, Phaneuf *et al.* 1994, Europe-Finner, Phaneuf *et*

al. 1997). This would result in increased PKA during pregnancy and a decrease during labour. The effect of these changes on the KCNMA1 channel would be dependent on the splice variant expressed at the different time points, and so a study specifically looking at STREX variant expression in the pregnant and labouring myometrium would be the next logical step in this research.

1.5.1.5 Regulation of KCNMA1 Expression

The KCNMA1 channel promoter has been cloned and characterised and this research has revealed two regions of the promoter which are necessary for positive promoter regulation. These regions are located -567bp to -220bp, and +80bp to +355bp from the Transcription Start Site (TSS). SP1 binding is thought to be responsible for the promoter activity in the -567bp to -220bp region as this region contains 3 SP1 binding sites (consensus sequence 5'-(G/T)GGGCGG(G/A)(G/A)(C/T)-3'; -331bp, -226bp, and -222bp) and deletion of this region decreased promoter activity. Whilst the +80bp to +355bp region is thought to contain positive regulatory elements as deletion of this region decreased promoter activity by 50% (Dhulipala and Kotlikoff 1999). The KCNMA1 promoter was also shown to contain a CCTCCC sequence which is located 27bp upstream of a TATA like sequence, this positioning is identical to that found in the SM1/2 gene and in this gene it results in increased transcriptional activity of the gene within smooth muscle (Dhulipala and Kotlikoff 1999). Two CArG boxes (consensus sequence 5'-$CC(A/T)_6GG$-3') were also identified at positions -361bp and -1449bp. A CArG box is a conserved sequence motif located in genes which comprise the MADS-box gene family of transcription factors, such as serum response factor (Schwarz-Sommer, Huijser *et al.* 1990). MADS-box genes are involved in a variety of functions such as muscle development and cell proliferation (Shore and Sharrocks 1995). Binding of serum response factor to CArG box chromatin has been shown to have a pivotal role in controlling the transcription of smooth muscle specific genes (McDonald, Wamhoff *et al.* 2006). This research, however, did not demonstrate specific transcription factor binding but rather inferred the importance of transcription factor binding due to the location of their binding sites in relation to the regions found to be required for positive promoter regulation. In addition to this hypermethylation of the KCNMA1 promoter has been shown to result in down regulation of transcription in pancreatic duct cells (Vincent, Omura *et al.* 2011).

1.5.1.6 Regulation of Splice Variant Expression

It is clear that the different splice variants allow the KCNMA1 channel to fine-tune its response to changing physiological conditions, but what triggers the expression of the different variants at the correct times is poorly understood. Estrogen and progesterone have been shown to affect the expression of the STREX exon. Estrogen elicits a reduction in STREX expression while progesterone causes an increase in expression (Holdiman, Fergus *et al.* 2002, Zhu, Eghbali *et al.* 2005).

A wide range of adrenal androgens and glucocorticoids have also been shown to affect STREX expression in chromaffin cells. Androgens seem to promote STREX expression while glucocorticoids inhibit its expression (Xie and McCobb 1998, Lai and McCobb 2002).

SVcyt is down regulated in diabetic mice, with an observed increase of up to 80 fold in the insert-less SV0 variant compared to the SVcyt variant. Treatment with insulin increased the expression of SVcyt (Davies, Zhao *et al.* 2007). The difficulty with this observation is that, as stated before, the SVcyt insert is comprised of three different inserts. Therefore it is necessary to clarify which of the variant sequences is key before conclusions can be drawn from this observation.

1.5.2 Voltage-Dependant Calcium Channels

These are a group of voltage-gated ion channels found in excitable cells that are permeable to calcium. They are voltage-dependant as they are activated at depolarized membrane potentials. Activation allows calcium to enter the cell which, depending on the cell type, results in muscular contraction, excitation of neurons, up-regulation of gene expression, or release of hormones or neurotransmitters. Voltage-dependent calcium channels are formed as a complex of several different subunits: α_1, $\alpha_2\delta$, $\beta_{1\text{-}4}$, and γ.

The α_1-subunit forms the ion-conducting pore and is comprised of four repeats (I – IV) with each repeat containing six membrane-spanning domains (S1 – S6 Figure 1.10).

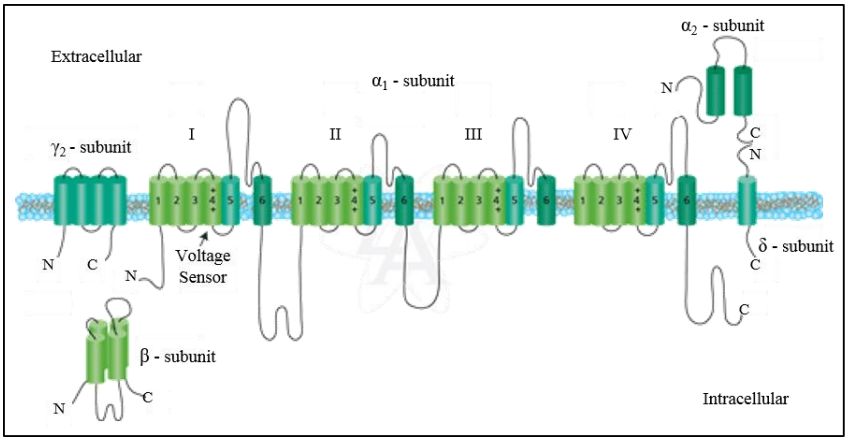


Figure 1.10 Voltage-depenant Calcium Channel
Subunit structures of a voltage-dependant Calcium Channel (Adapted from Sigma Aldrich)

There are several different kinds of voltage-dependant calcium channels: the neural N-type channel, the R-type channel involved in poorly defined processes in the brain, the closely-related P/Q-type channels, the CACNA1G involved in pacemaker activity and the CACNA1C responsible for excitation-contraction coupling of skeletal, smooth and cardiac muscle and for hormone secretion in endocrine cells.

1.5.2.1 Calcium Selectivity in the Voltage-Dependant Calcium Channel

The channel pore is comprised of a narrow external pore lined by the pore loop (Catterall 2000). The S5 and S6 membrane spanning domains and the membrane-associated linker region between them are responsible for the formation of the pore lining. Within the pore there are four glutamate residues, one in each repeat. Mutational analysis has shown that these glutamate residues are responsible for the high affinity the channel has for calcium (Ellinor, Yang *et al.* 1995).

1.5.2.2 Voltage Sensitivity in the Voltage-Dependant Calcium Channels

The S4 membrane spanning domains in each repeat serve as the voltage sensor for the channel (Yamakage and Namiki 2002). This region is comprised of repeating positively charged amino acids such as arginine or lysine in every third or fourth position. These positive residues function as gating charges. Mutational analysis of the positively charged residues found in the S4 segment of both potassium and sodium channels have shown that each positively charged residue does not contribute equally to the gating charge (Catterall 1995). When the membrane becomes depolarised, the S4 segment moves

outwards as a result of these positively charged residues and the pore opens (Reuter 1996, Catterall 2000)

1.5.3 The L-Type Calcium Channel (CACNA1C)

The L-type calcium channel is a type of voltage-dependent calcium channel. "L" stands for long-lasting referring to the length of activation. Five subunits (α1, α2, β, γ, and δ) make up the CACNA1C. Like the others of this class, the α1-subunit is the one that determines most of the channel's properties. They are responsible for normal myocardial and vascular smooth muscle contractility.

1.5.3.1. Splice Variants in the CACNA1C Calcium Channel

As with the KCNMA1 channel, the CACNA1C is also subject to splicing. Eleven sites of variation have been identified which span the whole length of the channel these are summarised in Table 1.2. Splice variants of the CACNA1C can affect functions as diverse as membrane expression, voltage dependence, calcium sensitivity, calcium dependent inactivation, excitation contraction coupling, β-subunit binding and sensitivity to DHP block (Perez-Reyes, Wei *et al.* 1990, Snutch, Tomlinson *et al.* 1991, Diebold, Koch *et al.* 1992, Soldatov 1992, Soldatov 1994, Yang, Chen *et al.* 2000, Yang, Obinata *et al.* 2000, Abernethy and Soldatov 2002, Tang, Liang *et al.* 2004, Bannister, Thomas-Gatewood *et al.* 2011).

Table 1.2 CACNA1C Calcium Channel Splice Variants

Splice variants founds in the human CACNA1C calcium channel, their location, exon profile and the effects they have on channel function

Locus	Channel Region	Splice Variation Profile	Putative Effects	References
L1	N-terminus	Mutually exclusive insertion of exon 1, 1b or 1c	Membrane expression	(Snutch, Tomlinson *et al.* 1991, Soldatov 1992, Bannister, Thomas-Gatewood *et al.* 2011)
L2	N-terminus / I S1	4 nucleotide insertion between exons 2 and 3	Unknown	(Snutch, Tomlinson *et al.* 1991, Soldatov 1992)
L3	I S4 – S5	12 nucleotide deletion between exons 6 and 7	Modification of calcium sensitivity	(Soldatov 1992, Abernethy and Soldatov 2002)
L4	I S6 - II	Inclusion of one of two mutually exclusive exons: 8 or 8a or the deletion of both, alongside the insertion or deletion of exon 9	Modification of dihydropyridine block sensitivity	(Soldatov 1992)
L5	Linking loop between I - II	inclusion or deletion of an exon: 9*	Production of non functional channel	(Tang, Liang *et al.* 2004)
L6	Linking loop between I - II	inclusion or deletion of an exon: 10*	Production of non functional channel	(Tang, Liang *et al.* 2004)
L7	II S4 –S5	73 nucleotide deletion	Modification of β subunit binding	(Soldatov 1992, Soldatov 1994, Abernethy and Soldatov 2002)
L8	Linking loop between II - III	12 nucleotide deletion	Modification of excitation / contraction coupling	(Snutch, Tomlinson *et al.* 1991)
L9	III S2	Inclusion of one of two mutually exclusive exons: 21 or 22 or the deletion of both	Modification of voltage dependence	(Soldatov 1992, Soldatov 1994)
L10	IV S2 – S4	There are twelve combinations of the 5 exons (30, 31, 32, 33, and 34) in this region.	Modification of voltage dependence, in relation to the length of the linking loop	(Perez-Reyes, Wei *et al.* 1990, Yang, Chen *et al.* 2000)
L11	C-terminus	52nt insertion	Modification of Calmodulin binding and calcium dependant inactivation.	(Perez-Reyes, Wei *et al.* 1990, Snutch, Tomlinson *et al.* 1991, Diebold, Koch *et al.* 1992, Soldatov 1992, Soldatov 1994, Tang, Liang *et al.* 2004).
L12	C-Terminus	Mutually exclusive insertion of exons: 45 or 45* with or without a 57 nucleotide deletion.	Removal of cAMP dependant protein Kinase A site	

It has also been shown that use of different splice variants, or combinations of splice variants, have a marked effect on current density, activation/inactivation kinetics and cell excitability (Snutch, Tomlinson *et al.* 1991, Soldatov 1992, Kepplinger, Kahr *et al.* 2000, Koschak, Reimer *et al.* 2001, Tang, Liang *et al.* 2004, Cheng, Pachuau *et al.* 2009, Bannister, Thomas-Gatewood *et al.* 2011). Temporal expression of specific variants could therefore be utilised to promote either relaxation or contraction depending on the needs of the cell. This ability to alter the physiological properties of the channel makes it an attractive target to study when examining the switch between myometrial quiescence and contractility. There is, however, a paucity of information regarding the control of expression of the different splice variants.

The ability of DHP to block CACNA1Cs is commonly utilised to quantify the number of channels in a particular cell or tissue sample. This could be responsible for giving researchers false information regarding the levels of CACNA1C expression, as there are variants with modified sensitivity to DHP block (Soldatov 1992). This highlights the importance of directly quantifying the level of channel expression and not relying solely on indirect methods.

Most studies have focused on a single splice variant and from this have inferred the specific variant's effect on the channels properties. However, this can give a distorted picture. It is unlikely that there is only one splice variant present and the expression of a combination of splice variants could have the effect of either amplifying the effects of individual splice variants or conversely they could reduce the effects of individual variants. This was demonstrated in a study by Cheng *et al.* where it was shown that the use of exon 33 with exon 9* leads to a negative shift in activation and inactivation potential, and inclusion of 33 without 9* leads to a positive shift in both the activation and inactivation potentials (Cheng, Pachuau *et al.* 2009).

Further studies by Soldatov *et al.* and Kepplinger *et al.* also demonstrate the cumulative effect of multiple splice variants. They both examined the region covering exons 39 – 43, which may modulate tethering of calmodulin to the C-terminal and therefore have an effect on calcium dependant inactivation of the channel (Soldatov, Zuhlke *et al.* 1997, Kepplinger, Kahr *et al.* 2000).

Three variants have been studied in this region $\alpha 1C_{86}$, $\alpha 1C_{72}$ and $\alpha 1C_{77}$. These three variants contain different complements of exons: $\alpha 1C_{86}$ contains exon 40 with a 17bp deletion, exon 40B and exon 43 with a 132bp extension; $\alpha 1C_{72}$ contains exon 40, exon 41, an additional 57bp, exon 42 and exon 43, $\alpha 1C_{77}$ contains exons 40, 41, 42, and 43.

It was found that $\alpha 1C_{86}$ had the fastest inactivation kinetics and recovery of the three variants. Both $\alpha 1C_{86}$ and $\alpha 1C_{72}$ were inactivated at more negative potentials than $\alpha 1C_{77}$ with $\alpha 1C_{86}$ inactivating at the most negative potential. Calcium-dependant inactivation was eliminated in $\alpha 1C_{86}$ and was highest in $\alpha 1C_{77}$ (Soldatov, Zuhlke *et al.* 1997). Also, $\alpha 1C_{77}$ was more efficiently targeted to the cell membrane and had higher conductance and open probability than $\alpha 1C_{86}$ (Kepplinger, Kahr *et al.* 2000).

It is possible that the poor targeting, low conductance and fast inactivation of the $\alpha 1C_{86}$ channel are protective measures due to the lack of calcium-dependant inactivation found in this channel. These would limit the amount of calcium the channel could allow into the cell.

Another point which is frequently overlooked when studying the CACNA1C is the fact that the channel is only active when it is inserted into the membrane. Clearly, however, there are splice variants which result in the channel being held in the cytoplasm in an inactive state. Hence, it is important to separate active membrane-bound channels from inactive cytosolic channels when measuring expression levels, especially if inferences are then made about the effect of channel expression on cell excitability. It would be interesting to examine if cytosolic retention of the CACNA1C is a reversible process as has been demonstrated with the Mk44 variant of the KCNMA1 channel, as this would open up the possibility of the cell being able to house a cytoplasmic store of CACNA1Cs which could be rapidly translocated to the cell membrane when required.

The different splice variants have been shown to affect smooth muscle contractility and so manipulation of the expression of these variants could hypothetically lead to repression of contractility, and may be of therapeutic use in the treatment of preterm birth. Hence, it is important to now focus on the physiological triggers for specific splice variant expression.

1.5.3.2 C-Terminal Proteolytic Cleavage of the CACNA1C Calcium Channel

The CACNA1C has been shown to exist in two forms: a long ~220kDa protein and a shorter ~190kDa protein. The shorter protein is formed by proteolytic cleavage of the C-terminal tail region. Calpain has been shown to be responsible for this cleavage (Hell, Westenbroek *et al.* 1996) which occurs in exon 42 between splice variants 11 and 12. Interestingly, it has been shown that calmodulin binds calpain substrates and in doing so can regulate calpain-mediated proteolysis causing inhibition of this process (Wang, Villalobo *et al.* 1989, Iwamoto, Lu *et al.* 2010). The 52 bp insertion at splice site 11 has been shown to modulate calmodulin binding and therefore this splice site could have an effect on the cleavage of the channel.

Once cleaved, the C-terminal fragment can either co-localise with the rest of the channel or can move into the nucleus (Hell, Westenbroek *et al.* 1996, Gerhardstein, Gao *et al.* 2000, Hulme, Konoki *et al.* 2005, Gomez-Ospina, Tsuruta *et al.* 2006, Hulme, Yarov-Yarovoy *et al.* 2006, Schroder, Byse *et al.* 2009, Satin, Schroder *et al.* 2011).

Cleavage of the C-terminus results in a calcium channel with four to six fold higher ion conductance in comparison with the full-length channel (Hell, Westenbroek *et al.* 1996), which would suggest that the C-terminal region exerts some form of inhibitory control. After proteolytic processing the C-terminal fragment associates non-covalently with the body of the channel and this interaction reduces the coupling of gating charge movement to channel opening, and the voltage dependence of the channel is moved to more positive membrane potentials (Hulme, Yarov-Yarovoy *et al.* 2006). This inhibition, however, is relieved by cAMP-dependant phosphorylation (Hulme, Konoki *et al.* 2005), due to the fact that phosphorylation of a putative PKA consensus sequence at serine 1,700 modifies the interaction of the C-terminal fragment with the body of the channel (Hulme, Yarov-Yarovoy *et al.* 2006). Again, there is a splice site in this location, site 12, where a 57 bp insertion removes a cAMP-dependant PKA serine site in exon 48. This gives another layer of control over channel activity.

Within the nucleus, the C-terminal fragment of the CACNA1C has been shown to bind to its own promoter and down-regulate transcription (Schroder, Byse *et al.* 2009, Satin,

Schroder *et al.* 2011). It has also been shown to regulate the expression of a number of genes including connexin 3.1 in neurons, the Na/Ca exchanger which works co-ordinately with the calcium channel to maintain cellular calcium (Schroder, Byse *et al.* 2009) and the potassium channel KCNN3 (Figure 1.11).

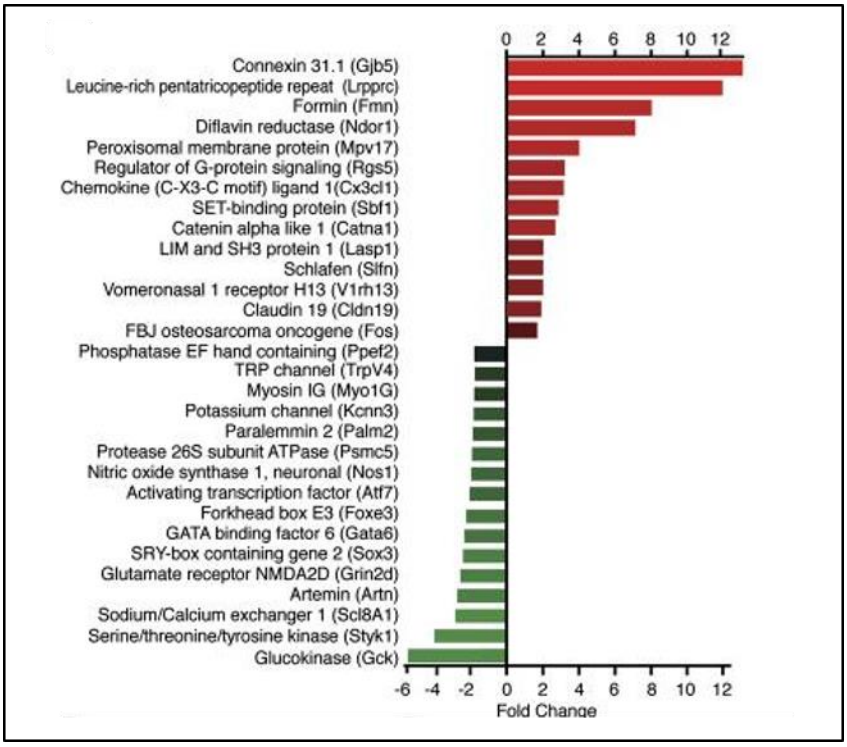


Figure 1.11 Changes in mRNA Expression due to CACNA1C C-terminal Fragment Expression
A subset of mRNAs identified in microarray experiments that were up regulated (red bars) or down regulated (green bars) by expression of the CACNA1C C-terminal proteolytically cleaved fragment (Reprinted with permission from *Gomez-Ospina, Tsuruta et al. 2006).*

C-terminal fragment nuclear localisation has been shown to be mediated by intracellular calcium, with decreasing calcium levels promoting nuclear localisation. Nuclear localisation has also been shown to be increased by serum-mediated cellular hypertrophy in cardiac myocytes (Gomez-Ospina, Tsuruta *et al.* 2006).

1.5.3.3 CACNA1C Calcium Channel Expression During Gestation

Information regarding expression levels of the CACNA1C protein or mRNA in the human myometrium during gestation is sparse. Parkington showed that there is an increase in myometrial cell excitability in the third trimester, which coincides with decreasingly negative membrane potentials. This has the effect of increasing the open probability of voltage-dependant calcium channels, which in turn provides the calcium

influx that is a critical component for both excitation-contraction coupling and for the phosphorylation of myosin light chains, which is necessary for their contraction (Parkington, Tonta *et al.* 1999).

Channel-blocking experiments have provided evidence that the majority of calcium currents are due to CACNA1Cs (Tezuka, Ali *et al.* 1995, Parkington, Tonta *et al.* 1999, Collins, Moore *et al.* 2000). The CACNA1C senses membrane depolarisation, opens and increases both intracellular calcium concentration and cell contraction.

Studies of rat myometrium have shown that the mRNA for the α-subunit of the CACNA1C channel increases gradually during gestation, peaking at around a 6.9 fold increase prior to labour then decreases sharply during labour and post-partum. This increase in the α-subunit is comprised of at least two splice variants, termed SIV3A and SIV3B. These two variants correlate well with some of the variants found within splice site 10. Splice site 10 occurs between IV S2 and S4 and involves the differential utilisation of exons thirty to thirty four. SIV3A correlates with the usage of exons 30, 31, 33 and 34 or 30, 31, 34 and SIV3B correlates with the usage of exons 30, 32, 33 and 34 or 30, 32, 34 (Mershon, Mikala *et al.* 1994). Prior to parturition the SIV3B splice variant is expressed in an approximately 2.5:1 ratio with the SVI3A variant. Then at parturition this ratio changes to a 10:1 ratio and post-partum the ratio reverts back to 2.5:1 (Mershon, Mikala *et al.* 1994). Huang *et al.* showed that in rabbit ventricular myocytes the SVI3B variant was associated with greatly increased excitation-contraction coupling (Huang, Xu *et al.* 2006). In the myometrium the increase in expression of this variant at parturition would support myometrial contractions.

Tribe *et al.* isolated two different sized α-subunit RNAs from myometrial tissue and showed that there was a greater increase in the longer α-subunit RNA compared to the shorter one (Tribe, Moriarty *et al.* 2000). Combining this information with the research by Mershon *et al.* related to the expression of the specific splice variants would indicate that the larger α-subunit RNA could correlate with the SIV3B variant, and the shorter α-subunit RNA could correlate with the SVI3A variant. These splice variants are in a region known to be involved in voltage dependence.

Voltage dependence has been shown to be linked to the length of the linking loop between repeat IV S3 and S4 (Perez-Reyes, Wei *et al.* 1990, Yang, Chen *et al.* 2000). The fact that Tribe *et al.* isolated two different sized α-subunit RNAs from the myometrial tissue would suggest that the splice variants would result in different sized proteins, and combining this information with the splice variants described by Mershon *et al.* would point to the fact that this difference in size could be specifically related to the IV S3-S4 linker region. These changes in α-subunit levels were found to be associated with both term and preterm induced labour.

Tezuka *et al.* demonstrated that if progesterone is used to prevent the α-subunit from reaching its peak levels then labour does not occur (Tezuka, Ali *et al.* 1995). In this research Onapristone, a progesterone receptor antagonist, is used to induce labour. As there is little understanding of the mechanisms by which a reduction in progesterone leads to the induction of parturition, it is difficult to ascertain just how accurate a picture of labouring myometrium this use of onapristone provides.

Both the regulation of expression of the CACNA1C and its functions would indicate that it could potentially play a significant role in the preparation of the myometrium for the switch from quiescence to contractility at labour.

1.5.3.4 Regulation of CACNA1C Calcium Channel Expression

The CACNA1C promoter is less well characterised, early research into the transcriptional regulation of the CACNA1C was carried out on the rat CACNA1C gene, in cardiac myocytes and vascular smooth muscle and this research identified a possible eight TSS and determined that the promoter did not contain a TATA box. This early research did, however, identify an initiator sequence spanning one of the TSS and so this was termed the major TSS. The region between -726bp and -234bp of the major TSS provided the majority of basal promoter activity (Liu, Fan *et al.* 2000). Later research revealed a >69% homology between the rat and human CACNA1C promoters (Dai, Saada *et al.* 2002). The CACNA1C, however, can utilise three different N-terminal sequences depending on the usage of either exon 1a, exon 1b or neither exon. Within smooth muscle it has been demonstrated that the majority of transcripts utilise exon 1b (Pang, Koren *et al.* 2003). Transcripts containing exon 1b have been shown to possess a unique 5’ untranslated

region, and the region immediately upstream of exon 1b was shown to have promoter activity suggesting that these exon 1b containing transcripts are regulated by a separate promoter (Saada, Dai *et al.* 2003). This putative exon 1b promoter (Promoter B) was also shown to be a TATA-less promoter. The absence of TATA or CAAT boxes alongside the presence of several GC-rich SP1 consensus elements mean that Promoter B is characteristic of a housekeeping-type promoter (Pang, Koren *et al.* 2003). Promoter B has been shown to contain a putative cAMP response element and binding of CREB and CBP have been shown to induce gene expression (Fan, Chen *et al.* 2000). Further interrogation of the Promoter B sequence revealed a putative CArG box sequence located +288bp from the TSS (Sun, Chen *et al.* 2006) and a CCTCCC sequence -597bp from the TSS, both of these elements are important for the regulation transcription of smooth muscle genes. Using the YAPP Eukaryotic Core Promoter Predictor tool a putative initiator sequence was located close to the TSS (Cartharius, Frech *et al.* 2005, Gershenzon and Ioshikhes 2005, Jin, Singer *et al.* 2006) and using the PROMO transcription factor binding motif search tool a TFII-I binding sequence was located +48bp from the TSS (Messeguer, Escudero *et al.* 2002, Farré, Roset *et al.* 2003), TFII-I has been shown to be important in initiating transcription from TATA-less promoters (Manzano-Winkler, Novina *et al.* 1996). Within TATA-less promoters the initiator sequence performs a similar function to the TATA box in that it co-ordinates the formation of a stable initiation complex which is necessary to facilitate the binding of RNA Polymerase II and the initiation of transcription (O'Shea-Greenfield and Smale 1992, Manzano-Winkler, Novina *et al.* 1996). As the CACNA1C promoter is CG-rich it is possible that methylation has a role in the regulation of transcription and within the heart hypomethylation of the CACNA1C promoter has been shown to increase gene expression (Koczor, Torres *et al.* 2015). Within colonic smooth muscle, however, although inflammation led to a suppression of the CACNA1C channel transcription, no changes in the methylation status of the promoter were observed (Choi, Chen *et al.* 2011). This would indicate that the effect of methylation on the CACNA1C promoter is tissue and / or stimulus specific.

1.5.4 The T-type Calcium Channel (CACNA1G)

The T-type calcium channel is another member of the voltage-gated calcium channel family. "T" stands for transient referring to the length of activation. As with other sub-types of voltage-gated calcium channel, the α1 subunit is the one that determines most of

the channel's properties. CACNA1G calcium channels may contain one of three α1 subunits, α1G (Cav3.1), α1H (Cav3.2) or α1I (Cav3.3; Nilius, Talavera et al. 2006, Talavera and Nilius 2006).

The CACNA1G calcium channel produces the pacemaker potential in the sino-atrial (SA) node of the heart (Mangoni, Couette *et al.* 2006). Similarly, in the central nervous system (CNS), CACNA1G calcium channels contribute to tonic bursting activity in the thalamus (Huguenard 1996). Both of these properties are potentially important in the generation of the rhythmic spontaneous contractions seen in the labouring myometrium.

1.5.4.1 Splice Variants in the CACNA1G Calcium Channel

Variants of the CACNA1G calcium channel are formed by the alternative use of six cassette exons (8, 14, 16, 26, 34 and 35), two alternative splice donor sites (25C and 30B), four alternative splice acceptors sites (25A, 25A', 25A'' and 31A) and one protein-coding intron (38B; Mittman, Guo *et al.* 1999, Chemin, Monteil *et al.* 2001, Bertolesi, Walia Da Silva *et al.* 2006, Emerick, Stein *et al.* 2006, Shcheglovitov, Vitko *et al.* 2008). These are summarised in Table 1.3.

Splicing of the CACNA1G has been shown to affect ion conductance, membrane expression, PKA phosphorylation sites and interaction with effector proteins (Chemin, Monteil *et al.* 2001, Bertolesi, Walia Da Silva *et al.* 2006, Emerick, Stein *et al.* 2006, Shcheglovitov, Vitko *et al.* 2008). Expression of these different variants results in channels with different activation/inactivation kinetics and firing patterns (Bertolesi, Walia Da Silva *et al.* 2006, Emerick, Stein *et al.* 2006). Splicing of this channel is mainly located in the cytoplasmic loop regions (Emerick, Stein *et al.* 2006, David, Garcia *et al.* 2010).

Splice variants have been described which are incapable of conducting ions due to the removal of differing amounts of the repeat II S6 helix (Chemin, Monteil *et al.* 2001, Bertolesi, Walia Da Silva *et al.* 2006, Emerick, Stein *et al.* 2006). If these variants are still inserted in the membrane as inactive channels, this then opens up the possibility that they could compete for accessory subunit or effector protein binding and hence have a negative effect on overall channel activity i.e. a dominant-negative inhibitor.

Table 1.3 CACNA1G Calcium Channel Splice Variants
Splice variants founds in the human CACNA1G calcium channel, their location, and their exon profile

Locus	Channel Region	Splice Variation Profile	Putative Effects	References
T1	Linking loop between I - II	Insertion or deletion of exon 8	Modification of protein trafficking	(Shcheglovitov, Vitko *et al.* 2008)
T2	Linking loop between II - III	Insertion or deletion of exon 14	Addition of a PKC Phosphorylation site	(Bertolesi, Walia Da Silva *et al.* 2006)
T3	Linking loop between II - III	Insertion or deletion of exon 16	Modification of interaction with effecter proteins	(Chemin, Monteil *et al.* 2001)
T4	Linking loop between III - IV	Three splice acceptors sites in exon 25 (25A, 25A', 25A'')	Non ion conducting channels	(Chemin, Monteil *et al.* 2001, Bertolesi, Walia Da Silva *et al.* 2006, Emerick, Stein *et al.* 2006)
T5	Linking loop between III - IV	Splice donor site in exon 25 (25C)	Modification of ion permeation and gating	
T6	Linking loop between III - IV	Insertion and/or deletion of exons 25C and 26	Modification of ion permeation and gating	
T7	Linking loop between IV S4 – S5	Splice donor site in exon 30 (30B)	Modification of voltage dependent activation	(Emerick, Stein *et al.* 2006)
T8	IV S5	Splice acceptor site in exon 31 (31A)	Prematurely truncated protein	(Emerick, Stein *et al.* 2006)
T9	C-terminus	Insertion or deletion of exon 34, 35 and protein coding intron 38	Modification of length and binding site complement of C-terminal Tail	(Bertolesi, Walia Da Silva *et al.* 2006).

As with the KCNMA1 and CACNA1C, the CACNA1G also has a variant which affects membrane expression. The fact that all three channels have variants which affect membrane expression would indicate that control of the channel in this way is evolutionarily important. It would be interesting to examine if the cytosolic retention of the CACNA1G is a reversible process as has been demonstrated with the Mk44 variant of the KCNMA1 channel, as this would open up the possibility of the cell being able to house a cytoplasmic store of CACNA1G which could be rapidly translocated to the cell membrane when required.

A number of studies have demonstrated that, as with the CACNA1C, expression of a combination of variants can have a cumulative effect on channels properties.

Studies of the effect of exons 14, 25C, 26, and 38B on channel properties have illustrated that inclusion of exon 25C generates a channel which is activated at the most negative

potentials and has the most negative steady state midpoint. Channels lacking both exons 25C and 26 are activated at more positive potentials and have the most positive steady state midpoints, and those expressing exon 26 activate at midpoint potentials and a mid-range steady state midpoint.

The expression of exon 14 with either exon 25C or 26 increases the rate of inactivation of these channels. The most rapid inactivation is seen with the combination of exons 14 and 25C. The expression of exon 14 without either exons 25C or 26 slows the inactivation of the channels.

In combination with either exons 25C or 26 with or without exon 14, the expression of exon 38B has no effect on inactivation. However, in the absence of all of these, exon 38B slows inactivation. Channels expressing exon 26 and with exon 38 absent have substantially increased steady state magnitude. However, the absence of exon 38 in the presence of exon 25C decreases steady state magnitude, and the absence of both exons 25C and 26 combined with the absence of exon 38 has no effect on steady state magnitude (Chemin, Monteil *et al.* 2001, Jagannathan, Punt *et al.* 2002, Bertolesi, Walia Da Silva *et al.* 2006, Emerick, Stein *et al.* 2006).

One set of variants worthy of further research are exons 34, 35 and 38. These are found in the C-terminal region which contains binding sites for calcium, calmodulin and $G_{\beta\gamma}$ proteins. Although functional studies are lacking for these variants, analogous variants in other CACNA1G have resulted in premature truncation, slowed activation, accelerated inactivation and slowed recovery from inactivation. These final three culminate in sustained firing patterns (Jurkat-Rott and Lehmann-Horn 2004), which are important in the generation of rhythmic contractions like those seen during labour.

Some of these variants have been shown to be developmentally regulated. For example, there is an almost global switch from exon 26 expression in the fetus to exon 25C expression in the adult (Emerick, Stein *et al.* 2006).

1.5.4.2 CACNA1G Calcium Channel Expression During Gestation

There is little direct information regarding CACNA1G protein or mRNA levels during gestation, although it has been reported that the expression levels of the CACNA1G vary in relation to gestational age, with a general increase throughout gestation (Fry, Sui *et al.* 2006, Blanks, Zhao *et al.* 2007, Lee, Ahn *et al.* 2009).

There is, however, electrophysiological evidence demonstrating the presence of these channels in the myometrium (Knock and Aaronson 1999, Fry, Sui *et al.* 2006, Blanks, Zhao *et al.* 2007, Shmygol, Blanks *et al.* 2007, Lee, Ahn *et al.* 2009, Aguilar and Mitchell 2010) and a large amount of information on the potential role of these channels in relation to myometrial contractility. Known CACNA1G functions could contribute to processes which are vital for myometrial smooth muscle contraction, control of resting membrane potential and increased intracellular calcium.

The myometrium is capable of generating spontaneous phasic contractions in response to rising intracellular levels of calcium independent of external stimuli (Tribe, Moriarty *et al.* 2000, Shmygol, Blanks *et al.* 2007, Lee, Ahn *et al.* 2009). The generation of these spontaneous phasic contractions is due to the ability of the cell to fire regenerative action potentials (Lee, Ahn *et al.* 2009). Regenerative action potentials are created when a channel opening at a low threshold is able to initiate the opening of enough channels to initiate an action potential.

One of the properties of the CACNA1G is that it opens upon small membrane depolarisations and allows calcium to enter the cell. This has the effect of further depolarising the cell membrane, which in turn allows the opening of other voltage dependent ion channels and initiates action potential bursts. Once the membrane has repolarised the CACNA1G deactivate slowly, which results in a significant calcium influx following an action potential.

This influx of calcium following the action potential is a potentially significant feature of the CACNA1G function as calcium is an important secondary messenger which, via myosin light chain kinase, is responsible for generating the force of contraction (Blanks, Zhao *et al.* 2007).

Transient hyperpolarisation of the cell membrane as a result of action potential bursts can also activate tonically inactivated CACNA1G. This results in more channels becoming available to open when the membrane next depolarises and increases their ability to generate further action potential bursts (Blanks, Zhao *et al.* 2007, Aguilar and Mitchell 2010). The net effect of this is an increase in intracellular calcium levels, which in turn could potentially have a significant role in the generation of the spontaneous phasic contractions seen in the myometrium (Lee, Ahn *et al.* 2009). Specific inhibition of the CACNA1G results in decreased force, frequency and amplitude of contractions, as well as a reduction in the magnitude of the initiation spike (Fry, Sui *et al.* 2006, Lee, Ahn *et al.* 2009, Aguilar and Mitchell 2010).

This evidence points to a role for calcium entry via CACNA1G in the initiation, frequency and force of myometrial contractions.

1.5.4.3 Regulation of CACNA1G Calcium Channel Expression

Two core promoters have been identified in the promoter region of the CACNA1G the first located between -43bp and +7bp (P_A) and the second located between +154bp and +204bp (P_B) from the TSS (Bertolesi, Jollimore *et al.* 2003). Sequential 5' deletions of the promoter region revealed a putative enhancer region located between -383bp and -231bp from the TSS as there was a decrease in promoter activity when this sequence was deleted. This enhancer region increased activity from the P_A promoter (Bertolesi, Jollimore *et al.* 2003). A second research group identified the region -105bp to the start ATG (+267bp to +372bp from the TSS) as important for strong activation of gene transcription, whilst they demonstrated that the region from -312bp to -280bp from the start ATG (-474bp to -442bp from the TSS) was responsible for negative regulation of gene transcription (van Loo, Schaub *et al.* 2012). It is possible, therefore, that the region between +267bp to +372bp may act as an enhancer for the P_B promoter. The region between the two promoters (+7bp to +154bp) and promoter P_B were shown to reduce the activity of the P_A promoter (Bertolesi, Jollimore *et al.* 2003). The activity of the two promoters was shown to be cell type specific (Bertolesi, Jollimore *et al.* 2003). Both CACNA1G promoters have the characteristics of a TATA-less core promoter and share sequence similarity to other initiator sequences. Within the promoters there are numerous binding sites for both SP1 and NFκB. As both promoters are TATA-less, SP1 may be

responsible for the recruitment of TATA-binding protein associated factors and may also facilitate the interaction between the basal transcription machinery and the core promoter (Bertolesi, Jollimore *et al.* 2003). Egr1 was shown to strongly activate CACNA1G transcription, the binding of Egr1 was shown to occur in the region between -1588bp and -1350bp from the TSS (van Loo, Schaub *et al.* 2012). Conversely, the first intron of the CACNA1G gene contains a highly conserved REST binding site, REST is a repressor element, binding of REST to the CACNA1G was shown to strongly counteract Egr1 activation of the CACNA1G (van Loo, Schaub *et al.* 2012). Methylation of the region between -300bp to -800bp from the start ATG in the CACNA1G was show to closely correlate with gene inactivation in colon cancer (Toyota, Ho *et al.* 1999), and so methylation may have a role to play in the regulation of transcription of the CACNA1G.

1.5.5 Accessory Proteins of the Voltage Dependant Calcium Channels

As stated previously, voltage-dependent calcium channels are formed as a complex of several different subunits: α_1, $\alpha_2\delta$ (CACNA2D1-4), $\beta_{1\text{-}4}$ (CACNB1-4), and γ (CACNG1-8). The binding of these accessory subunits to the core α-subunit can have a marked effect on channel kinetics, amplitude and trafficking.

1.5.5.1 Role of the Beta (β) Subunit (CACNB1-4)

The β-subunit binds with high affinity to the linker loop between repeats I and II in the α1-subunit (Bichet, Cornet *et al.* 2000, Dolphin 2003, Opatowsky, Chen *et al.* 2004, Van Petegem, Clark *et al.* 2004), and binding of this β-subunit has been shown to modify channel trafficking (Birnbaumer, Qin *et al.* 1998, Bichet, Cornet *et al.* 2000, Meir, Bell *et al.* 2000, Dolphin 2003, Opatowsky, Chen *et al.* 2004), voltage dependant activation and inactivation rates (Birnbaumer, Qin *et al.* 1998, Bichet, Cornet *et al.* 2000, Meir, Bell *et al.* 2000, Dolphin 2003, Van Petegem, Clark *et al.* 2004), current amplitude (Meir, Bell *et al.* 2000, Dolphin 2003), channel open probability (Opatowsky, Chen *et al.* 2004), G protein and kinase regulation (Bichet, Cornet *et al.* 2000, Meir, Bell *et al.* 2000), and both the number of drug binding sites and the affinity of drug binding (Bichet, Cornet *et al.* 2000, Hering 2002, Van Petegem, Clark *et al.* 2004).

The β-subunit binding site on the α1-subunit of the CACNA1C, the α-interaction domain (AID), is flanked by two endoplasmic reticulum retention signals, the first occurring 16aa

before the AID and the second 74aa after the AID (Bichet, Cornet *et al.* 2000). In the absence of the β-subunit these ER retention signals severely restrict surface expression of the channel. In the presence of the β-subunit, however, surface expression is increased, suggesting that binding of the β-subunit either masks the ER retention signal or facilitates the release of the channel from the ER (Bichet, Cornet *et al.* 2000). As the channel is only active once it is expressed on the cell surface this possibility of ER retention highlights the importance of separating active channel from inactive channel when examining the expression of the channel and inferring what effects these changes in channel expression may be having. Interestingly, the CACNA1G does not require the β-subunit for surface expression (Dolphin 2003).

Binding of the β-subunit causes a shift in the voltage dependence of activation and steady state inactivation in the hyperpolarising direction (Dolphin 2003). This results in channels which open at more negative membrane potentials and an increased number of channels opening. Overall, β-subunit binding creates channels with increased mean open time and decreased mean closed time (Dolphin 2003), which will increase intracellular calcium. This increased intracellular calcium is vital for myometrial contraction.

1.5.5.2 Role of the Alpha 2 Delta (α2δ) Subunit (CACA2D1-4)

The α2δ-subunit is composed of an extracellular α2 segment linked by disulphide bonds to a trans-membrane δ segment, which anchors the α2 segment (Gurnett, De Waard *et al.* 1996, Felix, Gurnett *et al.* 1997, Klugbauer, Marais *et al.* 2003).

Putative binding sites for the trans-membrane segment have been shown in both repeats III and IV in the link between S5 and S6 (Felix, Gurnett *et al.* 1997). However, a binding site has not been found in the α1-subunit of the CACNA1G (Klugbauer, Marais *et al.* 2003). Binding of the α2δ-subunit to the α1-subunit has been shown to modify channel kinetics (Klugbauer, Marais *et al.* 2003), voltage dependence of both activation and inactivation (Bichet, Cornet *et al.* 2000, Klugbauer, Marais *et al.* 2003), channel trafficking (Klugbauer, Marais *et al.* 2003), current amplitude (Gurnett, De Waard *et al.* 1996, Felix, Gurnett *et al.* 1997) and both the of number drug binding sites and the affinity of drug binding (Felix, Gurnett *et al.* 1997, Bichet, Cornet *et al.* 2000)

Co-expression of this subunit with the α1 / β-subunits form channels with accelerated activation and inactivation kinetics; this has been shown to be a function primarily of the δ segment. Co-expression also increased surface expression of the channel with a resulting 2-fold increase in current density in COS7 cells and this has been shown to be a function of the α2-subunit specifically (Klugbauer, Marais *et al.* 2003). COS7 cell are a fibroblast cell line and so care must be taken when extrapolating from this data into smooth muscle cells. An increase in current amplitude of around 10-fold has also been reported after binding of the α2δ-subunit (Gurnett, De Waard *et al.* 1996). It is unclear if these changes are a result of either improved targeting or stabilisation, or if the α2δ-subunit is having a direct modulatory effect. Overall, the binding of the α2δ-subunit will result in increased intracellular calcium and so again will promote myometrial contractility.

1.5.6 KCNMA1, CACNA1C and CACNA1G Channel Associations.

Caveola, specialist lipid rafts, are small invaginations of the plasma membrane (Anderson 1998). The presence of caveolae changes the local morphology of the plasma membrane and may play a role in excitation-contraction coupling (Calaghan and White 2006). They are formed and maintained primarily by caveolin proteins, namely caveolin-1, -2 and -3

(Daniel, El-Yazbi *et al.* 2006). Caveolin-1 and -3 can form discrete subcellular signalling compartments while caveolin-2 is thought to stabilise caveolae formed by caveolin-1 (Balijepalli, Foell *et al.* 2006). Caveolin-1 and -2 are ubiquitously expressed while caveolin-3 is smooth muscle specific (Capozza, Cohen *et al.* 2005, Kozera, White *et al.* 2009).

1.5.6.1 Caveola and Caveolins in the Myometrium

Caveolin-1 and -2 have been identified within the myometrium (Taggart, Leavis *et al.* 2000, Turi, Kiss *et al.* 2001, Smith, Babiychuk *et al.* 2005). Caveolin-3, however, appears to be difficult to detect. It has been reported as absent in mouse uterine biopsies (Li, Liu *et al.* 2001, Turi, Kiss *et al.* 2001) but reported as present both in rat uterine longitudinal smooth muscle and myocytic cells (Taggart, Leavis *et al.* 2000, Capozza, Cohen *et al.* 2005, Smith, Babiychuk *et al.* 2005). This confusion may arise through species specific differences in expression or in tissue specific differences in expression (i.e. between

longitudinal versus circular muscle in the uterus) this would mean that studies in different species or tissues could show different levels of expression.

The active KCNMA1 channel is located on the plasma membrane and can be found within caveolae (Grunnet and Kaufmann 2004, Brainard, Miller *et al.* 2005, Daniel, El-Yazbi *et al.* 2006). In the myometrium, KCNMA1 channels have been found to associate with caveolin-1 and -2 (Brainard, Miller *et al.* 2005, Lu, Alioua *et al.* 2006). The CACNA1C has also been co-localised to caveolae in canine airway smooth muscle (Darby, Kwan *et al.* 2000) and has been specifically co-localised with caveolin-3 in cardiac and skeletal muscle. The CACNA1G has also be co-localised with caveolin-3.

1.5.6.2 The Role Caveolae in Calcium Handling

Caveolae have been implicated in calcium handling (Darby, Kwan *et al.* 2000, Taggart, Leavis *et al.* 2000, Brainard, Miller *et al.* 2005). Within the myometrium it is thought that caveolae facilitate the localisation of key calcium handling components. Interestingly the SR is also closely associated with caveolae (Darby, Kwan *et al.* 2000) and the caveolae may have a role controlling the formation of local SR calcium release (Löhn, Fürstenau *et al.* 2000)

The structure of the calveolae creates a micro-domain which can 'house' a transient increase in calcium due to a caveola localised calcium channel, which in turn causes a local calcium release event (a calcium 'spark') from the closely associated SR (Löhn, Fürstenau et al. 2000; Figure 1.12). It is possible then for these calcium 'sparks' to occur without appreciable cytosolic calcium elevation.

1.5.6.3 The Hormonal Control of Caveolae and Caveolins

Recent work has revealed that caveolae may be under hormonal control, estrogen and progesterone have been shown to regulate both the number of caveolae and the levels of caveolins. Stimulus with estrogen reduced the number of caveolae by ~90% and, although progesterone had no effect on its own, when it was used in conjunction with estrogen it prevented this down regulation. A membrane bound form of estrogen receptor has been found localised within caveolae.

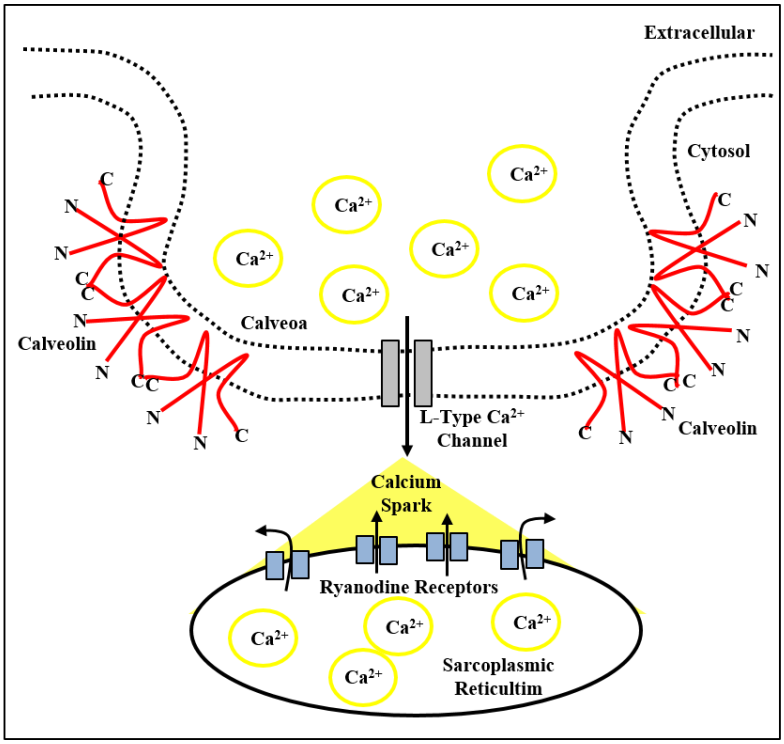


Figure 1.12 Schematic Representation of the Generation and Effect of Calcium Sparks
Calcium influx through calcium channels housed within caveolae can result in a localised calcium release event (calcium spark) from ryanodine receptors on closely associated sarcoplasmic reticulum this is termed calcium induced calcium release.

1.5.6.4 Role of Caveolae and Caveolins During Gestation

In rats it has been shown that during the first half of gestation caveolin-1 and -2 expression is suppressed to levels below that seen in non-pregnant controls. In the second half of pregnancy levels of caveolin-1 gradually increase until the day before parturition when they reach levels close to that seen in non-pregnant controls, while levels of caveolin-2 remain at around 25-30% of control levels throughout gestation (Turi, Kiss *et al.* 2001).

Cholesterol has also been shown to affect the formation of caveolae. During pregnancy there is a significant increase in cholesterol which increases the number of caveolae and inhibits uterine activity. This could indicate that caveolae are involved in uterine signalling cascades and possibly contractility. When cholesterol is reduced there is an increase in the force of contraction and calcium levels, which would indicate that caveolae use ion channels as mediators. This leads to the hypothesis that reduced cholesterol levels in pregnancy would result in an increased risk of preterm birth and conversely that high cholesterol levels would result in an increased risk of a prolonged labour due to the decreased force of the contractions (Smith, Babiychuk *et al.* 2005).

The β_2AR has been directly linked to both the CACNA1C in neurons (Davare, Avdonin *et al.* 2001, Liu, Shi *et al.* 2004), the KCNMA1 channel (Liu, Shi *et al.* 2004) and both channels together (Liu, Shi *et al.* 2004). This macromolecular complex (Figure 1.13) may also contain a G protein, an adenylyl cyclase, an A-kinase anchor protein and PKA (Chanrachakul, 2006). This complex would enable specific local regulation of both channels. Whether this complex exists within the caveolae, or whether this is a separate process by which the two channels can be brought into close proximity, is yet to be determined.

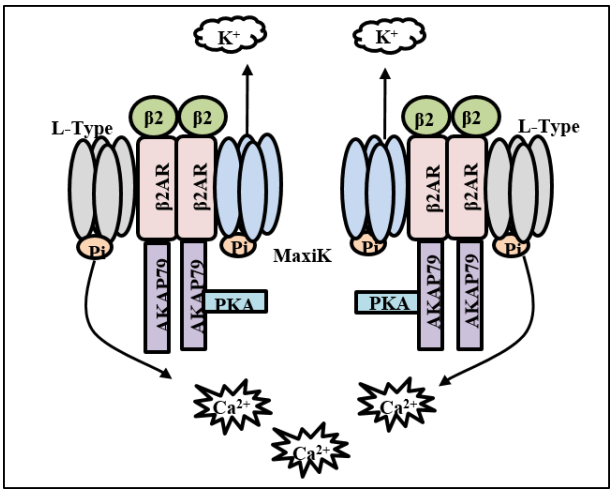


Figure 1.13 Proposed Macromolecular Complex
A molecular model of the proposed KCNMA1, CACNA1C, β_2 adrenergic receptor macromolecular complex (Adapted with permission from Liu, Shi *et al.* 2004)

Interestingly, some of the components of this complex are required for the maintenance of quiescence and are utilised as targets for current tocolytics For example, Ritodrine, a β_2AR agonist, targets the β_2AR on the outer membrane of myometrial cell, and activates AC which increases the level of cAMP, which in turn decreases intracellular calcium (Li, Zhang *et al.* 2005). Nifedipine, a calcium channel antagonists, targets calcium channels leading to a reduction in intracellular calcium. Both these lead to a decrease in uterine contractions. This may indicate that the formation of this complex is involved in the regulation of the timing of labour contractions.

1.6 Work Described in this Thesis

This project focuses primarily on the changes in the excitability of the myometrium as it progresses from quiescence to co-ordinated uterine contractions, and specifically the roles KCNMA1, CACNA1C and CACNA1G channels play in these changes.

The myometrium undergoes a number of changes during gestation and at the initiation of parturition. The most obvious of these is suppression of contraction during gestation and the generation of strong rhythmic contractions during labour. As well as these changes, there are a number of less obvious modifications including an increasingly depolarised plasma membrane, an increase in intracellular calcium, increased myometrial cell excitability and increased levels of cholesterol, estrogen and progesterone. In mice there is also a decrease in progesterone around the onset of parturition. This decrease in progesterone is not seen in humans and so a different mechanism may be in place.

To date it has been shown that all three channels (KCNMA1, CACNA1C and CACNA1G) can be modulated through the expression of splice variants with differing calcium and voltage sensitivities and gating kinetics. This would suggest that these ion channels play a central role in both the maintenance of quiescence and in the generation of contractions at parturition.

Quiescence could be initiated and maintained through the co-ordinated expression of KCNMA1 variants, with increased activation kinetics and increased calcium sensitivity such as the Ca27 and / or the STREX negative variant and high β-subunit expression. Alongside the expression of CACNA1C variants with slower activation kinetics, increased calcium dependant inactivation and reduced membrane expression such as variants containing exons 1c, 8, 19 or 17 and 18 and / or exon 33 and reduced β-subunit expression.

Towards the end of gestation, contraction could be initiated through a shift in the splice variant expression. At this time KCNMA1 channels with low calcium sensitivity such as the Mk44 and STREX insert channels could be expressed which would promote contraction. At the same time expression of CACNA1Cs with faster activation kinetics, improved membrane targeting and decreased calcium dependant activation such as

variants containing exon 1, 8*, 9* and possibly variant $\alpha 1C_{86}$ would also promote contraction. Increased expression of the CACNA1G channel variants which promote sustained firing would help maintain contractions.

Temporal control of expression of different ratios of channel splice variants could have a significant role in both the maintenance of quiescence and in the initiation and maintenance of contractility. Splicing requires the formation of the spliceosome complex. The spliceosome is formed in the following way: U1 binds to the GU site in the pre-mRNA, SF1 binds to the 'A' branch site and U2AF binds to the pyrimidine rich sequence. Then, following ATP hydrolysis SF1 leaves the complex and U2 binds to the 'A' branch site. Following this the U4/U6·U5 tri snRNA is incorporated into the complex and links U1 and U2. Finally the complex undergoes rearrangement, where U6 replaces U1 at the GU site, to form a catalytically active complex in which U2 and U6 interact. The formation of this complex is regulated by splicing factors which bind to splicing regulatory elements and either enhance or silence splicing. Serine-Arginine rich proteins are splicing factors which recruit the splicing machinery to splice sites while heterogeneous nuclear ribonucleoproteins are splice factors which repress splicesome assembly.Within the myometrium it has been demonstrated that splice factors Splice factor 2 (SF2) and hnRNPA1 are controlled in a spacio-temporal fashion (Pollard, Sparey *et al.* 2000, Shin and Manley 2004). SF2 is a transacting splicing factor which is necessary for all splicing to occur, as it promotes the recruitment of U1 to the splicesome while hnRNPA1 is a splicing suppressor, which represses spliceosome assembly by multimerization, blocking the binding of the splicing machinery.

Pollard *et al.* demonstrated that during gestation there was a substantial increase in SF2/ASF levels concomitant with a decrease in the levels of hnRNPA1/$A1^B$ in the lower uterine region. This pattern of expression was shown to be reversed in the upper uterine region (Pollard, Sparey *et al.* 2000). During labour there was a further increase in hnRNPA1/$A1^B$ in the upper uterine region and a significant decrease in the levels of SF2/ASF in the lower uterine region. Pollard *et al.* hypothesised that this polariation of expression could regulate the spacial expression of specific protein isoforms (Pollard, Sparey *et al.* 2000). This specific spacial protein isoform expression may be important in defining the functions of the different uterine regions e.g. the upper region governs contraction whilst the lower region governs dilation.

Tyson-Capper *et al.* also studied the role of splicing in the regulation of uterine activity, specifically the regulation of the splicing of Cyclic AMP-response Element Modulator (CREM) protein (Tyson-Capper, Bailey *et al.* 2005). CREM has a major role in the regulation of cAMP-responsive genes which are involved in uterine activity during gestation. CREM can exist as two isoforms: CREM$\tau_2\alpha$, a potent transcriptional activator and CREMα, a transcriptional repressor. The expression of these isoforms is regulated by SRp40. Tyson-Capper *et al.* showed that SRp40 was down-regulated during pregnancy which lead to the expression of the CREMα isoform and hence a repression of cAMP-responsive genes (Tyson-Capper, Bailey *et al.* 2005). This previous research demonstrates the potentially important role splicing has in the regulation of uterine function.

Functional analysis has revealed potential Exonic Splicing Enhancer (ESE) motifs for SF2 within the CACNA1C (Beitelshees, Navare *et al.* 2009). Given the similarities between the channels, it is possible that the KCNMA1 and CACNA1G may also contain ESE motifs for SF2. Further investigation of the role of channel splice variant expression and its role in the regulation of the myometrium during gestation and parturition is necessary.

The hypothesis is therefore:

Expression of the KCNMA1, CACNA1C and CACNA1G channels and / or specific splice variants, alongside their sub-cellular localisation regulates the switch between quiescence and contractility at the end of pregnancy

The aims of this project are to:

1 To establish myometrial cell cultures as a model system to examine the role of ion channels in the switch between relaxation and contractility.

2 To measure the expression levels of the KCNMA1, CACNA1C and CACNA1G channels and to identify which splice variants are expressed within the model system.

3 To define the sub-cellular localisation of the KCNMA1, CACNA1C and CACNA1G channels within the model system.

4 To identify which transcription factors are utilised in the expression of the KCNMA1, CACNA1C and CACNA1G channels.

5 To identify protein interactions between the KCNMA1 and CACNA1C channels and the components of the proposed macromolecular complex discussed in Section 1.5.6.4, namely the β_2Adrenergic receptor, $G_{\alpha s}$ and Caveolin-1.

6 To determine if the results from aims 2-5 are affected by altering the contractility of the myometrial cell cultures. TNF will be used to induce contraction and Trichostatin A (TSA) will be used to induce relaxation. TNF has previously been shown to induce contraction in myometrial smooth muscle strips, however the mechanism by which it achieved this is still unclear (Webster, Waite *et al.* 2013). TSA, a pan class I/II histone deacetylase (HDAC) inhibitor, has previously been shown to affect the expression the myometrial $G_{\alpha s}$ gene with subsequent effects on contractility but can also affect this activity independent of chromatin remodelling via regulating non-nuclear acetylation of contractile proteins as observed by Moynihan *et al.* (Moynihan, Hehir *et al.* 2008) and Karolczak-Bayatti *et al.* (Karolczak-Bayatti, Sweeney *et al.* 2011).

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 General Materials Reagents and Enzymes

The reagents used for the following procedures were obtained from Fisher (Loughborough. Leics., U.K.), Sigma (Poole, Dorset, U.K.) and VWR International Ltd. (Lutterworth, Leics., U.K.). iScript cDNA synthesis kits were purchased from Bio-Rad Laboratories (Hercules, CA, USA). PCR Master Mix and DNA loading dye was purchased from Promega and primers from Eurofins MWG Biotech (London, U.K.). Herculase II Fusion polymerase and dNTPs were purchased from Agilent (Stockport, Cheshire, U.K.). All cell culture plastic-ware was supplied by Sarstedt (Leicester, U.K.). Fetal Calf Serum (FCS) was purchased from Lonza (Wokingham, U.K.) and DMEM D-valine medium was purchased from PAA (Yeovil, Somerset, U.K.). Channel antibodies for the KCNMA1 (APC-021), CACNA1C (ACC-003) and CACNA1G (ACC-021) channels were purchased from Alomone Labs (Jerusalem, Israel). Antibodies for NFκB RelA (sc-372), CBP (sc-25748), MZF (sc-66991), SP1 (sc-14027), KCNMA1 Channel N-terminus (sc-14746) and CACNA1C channel C-terminus (sc-16230) were purchased from Santa Cruz Biotechnology Inc. (CA, USA), KCNMA1 channel N-terminus (ab104624) and CACNA1C channel C-terminus (ab140766) for use in western blotting were purchased from Abcam (Cambridge, U.K.), NFκB p50 (ab1602) from Millipore (Watford, U.K.) goat anti-rabbit and rabbit anti-goat HRP-conjugated secondary antibodies were purchased from Dako (Ely, Cambs, U.K.). All staining reagents were purchased from Vector Laboratories (CA, USA) excluding the antibody diluent which was purchased from Dako (Ely, Cambs, U.K.). ChIP reagents were purchased from Millipore (Watford, U.K.). Subcellular protein fractionation kit was purchased from Pierce Biotechnology (Rockford, USA). MeDIP reagents were purchased from Epigentek (Farmingdale, NY, USA).

2.1.2 Apparatus

All capital laboratory equipment marked (*) was purchased with funding from the MRC (Grant No. 84891 to Dr N. R. Chapman)

Equipment	Model and Supplier
Freezers	-80º New Brunswick Scientific Innova U535, Wolflabs* -20º Freezer GG5210, Fisher*
Fridges	4º Fridge Liebherr sparkfree, Wolflabs* Chromatography Cabinet, Wolflabs*
Centrifuges	Eppendorf Centrifuge 5415R Sigma 1-14 Sigma 1-16 Rotor
DNA Electrophoresis	Geneflow Multisub Midi, Geneflow Multisub mini
Gel Documentation	Syngene G:Box iChemi, Syngene Genetools version 4 quantification software Dell Vostro 1510
Homogeniser	IKA Ultra Turrax T25
Incubators	Thermo Scientific Hera Cell 240 Sanyo CO_2 Incubator MCO-18AIC
PCR Machine	SensoQuest Gradient Thermalcycler
pH Meter	Jenway 3020 pH Meter
Power Pack	Geneflow MP-250N
Protein Blotting	Bio-Rad Trans-Blot™ SD Semi-Dry Transfer cell Geneflow OmniPage Blot Mini
Protein Electrophoresis	Bio-Rad Mini-protean® Tetra Cell
Rocking Platform	Stuart
Shaking incubator	Stuart Orbital Incubator S1500
Sonication	Sonics Vibra Cell
Spectrophotometer	Nanophotomer (Implen)
UV Transilluminator	TFX-20M, Vilber Lourmat
Water Purification	Ultra Clear TWF UV Plus, SG Wasseraufbereitung und Regenerierstation GmbH Germany.

2.1.3 General Buffers used

Buffer	Composition
SDS-PAGE 4X Lower Buffer	1.5M Tris pH 8.8 and 0.4% (w/v) SDS
SDS-PAGE 4X Upper Buffer	0.5M Tris pH 6.8 and 0.4% (w/v) SDS
SDS Loading Buffer	Prepared to a final volume of 24ml: 3.0ml 500mM Tris pH 6.8, 2.4ml glycerol, 4.8ml 10% (w/v) SDS, 1.2ml β-mercaptoethanol, 600µl 0.05% (w/v) bromophenol blue and 12.0ml MilliQ water (18 MΩ)
Towbin Transfer Buffer (TTB)	25mM Tris pH 7.4, 192mM Glycine and 20% (v/v) Methanol
Tris Glycine Buffer (TGB)	25mM Tris, 192mM Glycine, 0.1% (w/v) SDS and 10% (v/v) Methanol
10X Electrophoresis Buffer	Prepared to a final volume of 1L: 250mM Tris, 520mM Glycine, 10% (w/v) SDS.
Phosphate buffered Saline pH 7.3 (PBS)	137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.47mM KH_2PO_4
Phophate buffered Saline (Tween 20) pH 7.4 (PBS-T)	As PBS but including 0.05% (v/v) Tween-20
Tris buffered Saline (TBS)	50mM Tris-Cl, pH 7.6, 150mM NaCl
Tris buffered Saline (Tween 20) (TBS-T)	As TBS but including 0.1% (v/v) Tween-20
Membrane Blocking Buffer Milk (TBS-TM)	As TBS-T but including 10% (w/v) low fat dried milk powder (Marvel, Premier Foods)
Tris Acetate EDTA (TAE)	Prepared as a 50X stock concentrate: 242g Tris, 57.1ml glacial acetic acid and 100ml 500mM EDTA pH 8.0
Whole Cell Lysis Buffer	150mM Sodium Chloride, 0.5% (v/v) Sodium Deoxycholate, 0.1% (v/v) SDS, 50mM Tris pH 8.0 and 0.1% (v/v) Igepal

2.2 Cell Culture Methods

2.2.1 Myometrial Biopsy Collection

The myometrial cell cultures utilised in this Thesis had been previously derived from myometrial biopsies obtained under ethical approval by the Rotherham Local Research Ethics Committee (Ref No. 05/Q2306/22). This favourable review was renewed by the Leeds Bradford Research Ethics Committee in 2012 (Appendix 2; Ref No. 12/YH/0229). Use of these previously derived cultures ensured women were not subjected to any unnecessary surgical procedures.

For these previously derived cultures, all women were recruited at the Department of Obstetrics and Gynaecology at the Jessop Wing Hospital for Women, Sheffield.

Lower segment myometrial biopsies were taken from women undergoing elective Caesarean sections at term. The lower segment biopsies were taken from the upper lip of the incision using tooth biopsy forceps and curved scissors and placed immediately into D-valine-DMEM pre-chilled to 4ºC.

2.2.2 Cell Culture Techniques

2.2.2.1 Generation of Myometrial Cells

Biopsies were collected in D-valine medium and washed in PBS to remove excess blood. A collagenase mix containing 10mg collagenase, 0.2mg DNase, 2mg elastase, 2mg trypsin inhibitor and 2mg Bovine Serum Albumin (BSA) was prepared in a sterile tube, and was diluted with 10ml PBS immediately prior to use. This was then sterilised using a 0.22µm syringe filter. The biopsy was minced into $3mm^3$ pieces and added to the collagenase mix and incubated in a shaking incubator (80rpm) at 37ºC for three to four hours. The debris was then allowed to settle under gravity. The supernatant was transferred to a fresh sterile tube and centrifuged at 500*g* for ten minutes. The pellet was then resuspended in D-valine medium supplemented with 10% (v/v) FCS, 2mM L-glutamine, 100U/ml penicillin and 0.1mg/ml streptomycin and placed into a $25cm^2$ tissue culture flask. After one hour the cell supernatant, containing the myocytes, was removed and placed into a fresh $25cm^2$ tissue culture flask and maintained in a humidified atmosphere at 37ºC and 5% CO_2. The replacement of L-valine with D-valine in the culture

medium selectively inhibits fibroblast proliferation as fibroblasts are unable to metabolise D-valine (Gilbert and Migeon 1975). After 24 hours the medium was replaced with fresh supplemented D-valine medium. A single biopsy was used to generate each individual cell culture.

2.2.2.2 Myometrial Cell Passaging

The cells were passaged on reaching 90% confluence and expanded from a 25cm^2 flask to a 75cm^2 flask. When confluent the 75cm^2 flask was expanded one to three etc. Cells were passaged by discarding the medium, washing the cells briefly with sterile PBS containing 1mM EDTA in order to remove any traces of serum prior to the addition of trypsin (6U/ml), 3ml of trypsin was added and the flask incubated at 37ºC for five minutes. The detached cells were then gently dispersed by pipetting and then redistributed into flasks containing fresh medium.

2.2.2.3 PHM1-31 Cell Passaging

PHM1-31 cells were a kind gift from Prof. Barbara Sanborn, Colorado State University, USA (Monga, Ku *et al.* 1996). Cells were maintained in DMEM medium supplemented with 10% (v/v) FCS, 2mM L-glutamine, 100U/ml penicillin, 0.1mg/ml streptomycin and 0.1mg/ml geneticin (Invitrogen). Passaging was performed as described above. PHM1-31 cells were used as a myocyte positive control during molecular characterisation of primary myocyte cultures.

2.2.2.4 Oral Fibroblast Cell Passaging

Primary oral fibroblast cells were a kind gift from Vannessa Hearnden, Dental School, University of Sheffield. Cells were maintained in DMEM medium supplemented with 5% (v/v) FCS, 2mM L-glutamine, 100U/ml penicillin and 0.1mg/ml streptomycin. Passaging was performed as described above. Fibroblasts were used as a fibroblast positive control during molecular characterisation of primary myocyte cultures.

2.3 Immunocytochemistry Techniques

2.3.1 Immunocytochemical Staining for the KCNMA1, CACNA1C and CACNA1G Channels.

Myometrial cells were cultured in a six-well plate. Upon reaching 80% confluence the cells were stimulated with 10ng/ml TNF for one hour, or 2µg/ml TSA for 24 hours with non-stimulated wells serving as a control. The cells were then fixed in 1% (v/v) formaldehyde overnight at 4ºC. Prior to staining the cells were washed three times for five minutes in PBS and permeabilised with PBS containing 1% (w/v) BSA and 0.1% (v/v) Triton X-100 with three, five minute washes. After rinsing again in PBS, endogenous peroxidase was quenched with 3% (v/v) hydrogen peroxide for 10 minutes. The Vectastain® *Elite* ABC Kit was used for the following reactions. Non-specific background was blocked with PBS containing goat serum and avidin for one hour at room temperature. The block was removed and the primary antibody was diluted in antibody diluent and biotin and incubated overnight at 4ºC. The cells were washed in PBS and the secondary anti-mouse IgG added to each well for 30 minutes at room temperature. After washing, the ABC reagent was added for 30 minutes at room temperature. Excess was removed by washing with PBS and the AEC chromagen substrate added for 10 minutes at room temperature. Cells were stored in PBS and photographed.

Antibody dilutions were as follows:

	Antibody Dilution for:		**Antibody used for Immunoprecipitation**
Antibody	**Immuno-cytochemistry**	**Western Blot**	
Anti-BK_{Ca}	1:200	1:2,000	-
Anti-$Ca_v1.2$	1:300	1:4,000	2.5µg
Anti-$Ca_v3.1$	1:200	1:4,000	-
L-Type Ca^{++}CP α1C	1:50	1:3,000	-
Anti-MaxiK K^+ channel α	-	1:5,000	2.5µg

2.4 Collagen Gel Contraction Assay

2.4.1 Preparation of Collagen for use in Collagen Gels

A 0.2% (v/v) acetic acid solution was prepared and filter sterilised using a 0.2µm filter. This was then added to the collagen to prepare a 6mg/ml collagen solution; this solution was gently agitated for five days at 4ºC to ensure the collagen was completely in solution. Once the collagen was completely dissolved the solution was diluted with an equal volume of filter sterilised water to produce a 3mg/ml collagen solution. This was gently agitated for a further day at 4ºC to equilibrate the solution.

2.4.2 NaOH Titration of Collagen

In order to optimise the solidification of each batch of collagen it was necessary to carry out a titration in order to identify the optimal amount of NaOH to add to the collagen medium mixture. Briefly, 0.4ml DMEM medium was added to each of eight microcentrifuge tubes. Then to each tube in turn 0.2ml 3mg/ml collagen was added, followed immediately by 1.0µl 1M NaOH. This mixture was pipetted up and down three times then left to solidify for 20 minutes. This process was repeated across all eight tubes using increasing amounts of 1M NaOH (1-8µl).

The rigidity and colour of the resulting gels were then compared, to determine the lowest volume of NaOH required to produce the most rigid of gels, whilst turning the phenol red indicator a pale pink colour.

2.4.3 Pouring Populated Collagen Gels

Trypsin (3mls) was added to a confluent 75 cm^2 flask of myometrial cells and the flask incubated at 37ºC for five minutes. The detached cells were then gently dispersed by pipetting and then 270µl of suspended cells were transferred to a 15ml centrifuge tube and 3.33mls of fresh media added. This cell suspension was then split into 400µl aliquots. Then to each of these aliquots in turn 200µl of 3mg/ml collagen was added along with the previously optimised quantity of NaOH. This mixture was pipetted up and down three times then 500µl transferred to each well of a 24 well plate and left to solidify for 20min. Finally a further 500µl of cell culture medium was added to each well.

2.4.4 Collagen Gel Contraction Assay

Upon reaching 80% confluence the collagen gel was dissociated from the sides of the well by running the tip of a 200µl pipette tip around the edges of the gel. Then the plate was gently swirled to ensure the gel was free floating. The myometrial cells were then stimulated with 10ng/ml TNF, or 2µg/ml TSA, or 10mg/ml Oxytocin or left un-stimulated. PHM1-31 cells were used as contraction positive controls while HEK293 cells were used as contraction negative controls. At pre-determined time points the plate was removed from the incubator for image acquisition. Images were captured using the Syngene G:BOX Chemi-16 gel documentation system. The Syngene Genetools Version 4 quantification software (SynGene, Cambridge UK) was used to trace the outline of the gel at each time point and to calculate the mean pixel area. The surface area at each time point is reported as a percentage of the initial gel surface area in that well, a decrease in surface area indicates contraction whilst an increase indicates relaxation.

2.5 Protein Methods

2.5.1 Whole Cell Lysis (WCL) of Cultured Myocytes

Confluent 75 cm^2 flasks of cultured myocytes were stimulated with 10ng/ml TNF or, 2µg/ml TSA or, 10ng/ml TNF followed by 2µg/ml TSA or, 2µg/ml TSA followed by 10ng/ml TNF with a non-stimulated 75 cm^2 flask serving as a control. The medium was removed and the cells washed with ice cold PBS. Then 500 µl of ice cold whole cell lysis buffer containing protease inhibitors (Complete Mini, Roche) was added to each flask. Cells were scraped off the flask and transferred to a fresh pre-cooled microcentrifuge tube and then incubated for 30 minutes at 4ºC on a roller. Following this incubation the lysate was centrifuged in a pre-cooled microcentrifuge tube for 20 minutes at 11,400*g* at 4ºC. Protein was quantified using the nanophotometer (Implen, Germany) by measuring UV absorbtion at 280nm, amino acids with aromatic rings primarily absorb at 280nm. Using the Beer Lambert Law where an absorbtion of one equates to a concentration of 90µg/ml protein. The ratio of UV absorbtion at A260 and A280nm was used to assess the purity of the sample. The $A_{260/280}$ for protein is between 0.5 and 1.5.

2.5.2 Sequential Extraction of Protein from Cultured Myocytes

Confluent 75cm^2 flasks of cultured myocytes were stimulated 10ng/ml TNF or, 2µg/ml TSA or, 10ng/ml TNF followed by 2µg/ml TSA or, 2µg/ml TSA followed by 10ng/ml TNF with a non-stimulated 75 cm^2 flask serving as a control. Sequential extraction was performed using Pierce Subcellular Protein Fractionation Kit according to the manufacturers' guidelines. Briefly, the medium was removed and the cells washed with ice cold PBS. Then 500µl of ice cold PBS with protease inhibitors was added to each flask. Cells were scraped off the flask and transferred to a fresh pre-cooled microcentrifuge tube and then centrifuged at 500*g* for five minutes. The supernatant was removed and 200µl cytoplasmic extraction buffer was added to the cell pellet and incubated on a roller for ten minutes at 4ºC. The lysate was then centrifuged at 500*g* for five minutes and the supernatant transferred to a fresh pre-cooled microcentrifuge tube, this is the cytoplasmic fraction. The cell pellet was then resuspended in 200µl membrane extraction buffer, vortexed vigorously for five seconds then incubated on a roller for ten minutes at 4ºC. The lysate was then centrifuged at 3,000*g* for five minutes and the supernatant transferred to a fresh pre-cooled microcentrifuge tube, this is the membrane fraction. The cell pellet was then resuspended in 100µl nuclear extraction buffer, vortexed vigorously for 15 seconds then incubated on a roller for 30 minutes at 4 ºC. The lysate was then centrifuged at 5,000*g* for five minutes and the supernatant transferred to a fresh pre-cooled microcentrifuge tube, this is the soluble nuclear fraction. The cell pellet was then resuspended in 100µl nuclear extraction buffer containing 5mM $CaCl_2$ and 300 units Micrococcal Nuclease, vortexed vigorously for 15 seconds then incubated at room temperature for 15 minutes. The lysate was then vortexed vigorously for 15 seconds and centrifuged at 16,000*g* for five minutes and the supernatant transferred to a fresh pre-cooled microcentrifuge tube, this is the chromatin bound nuclear fraction. The cell pellet was then resuspended in 100µl pellet extraction buffer, vortexed vigorously for 15 seconds then incubated at room temperature for ten minutes. The lysate was then centrifuged at 16,000*g* for five minutes and the supernatant transferred to a fresh pre-cooled microcentrifuge tube, this is the cytoskeletal fraction. Protein was quantified using the nanophotometer (Implen, Germany) by measuring UV absorbtion at 280nm, amino acids with aromatic rings primarily absorb at 280nm. Using the Beer Lambert Law an absorbtion of one equates to a concentration of 90µg/ml protein. The ratio of UV absorbtion at A260 and A280nm was used to assess the purity of the sample. The $A_{260/280}$ for protein is between 0.5 and 1.5.

2.5.3 Co-Immunoprecipitation of Proteins

2.5.3.1 Lysis of cell Monolayer

Confluent 75cm^2 flasks of cultured myocytes were stimulated with 10ng/ml TNF or 2μg/ml or TSA for 24 hours with a non-stimulated 75 cm^2 flask serving as a control. Cell lysis was performed using Pierce Crosslink Magnetic IP/Co-IP Kit according to the manufacturers' guidelines. Briefly, the media was removed from the flasks and the cells washed with PBS. Seven hundred and fifty microliters of ice cold IP lysis/wash buffer was added to each flask. The flasks were then incubated on ice for five minutes with periodic mixing. The lysate was then transferred to a microcentrifuge tube and centrifuged at 13,000*g* for ten minutes to pellet the cell debris. Finally the supernatant was transferred to a new microcentrifuge tube and the protein quantified quantified using the nanophotometer (Implen, Germany) by measuring UV absorbtion at 280nm, amino acids with aromatic rings primarily absorb at 280nm. Using the Beer Lambert Law where an absorbtion of one equates to a concentration of 90μg/ml protein. The ratio of UV absorbtion at A260 and A280nm was used to assess the purity of the sample. The $A_{260/280}$ for protein is between 0.5 and 1.5.

2.5.3.2 Binding of Antibody to Protein A/G Beads

Briefly, 2ml of 1X coupling buffer (10mM sodium phosphate, 150mM NaCl; pH 7.2) was prepared for each reaction by diluting 0.1ml 20X coupling buffer and 0.1ml IP lysis/wash buffer in 1.8ml ultrapure water. The protein A/G beads were vortexed and 25μl added to a microcentrifuge tube per sample. Using a magnetic stand the beads were collected and the storage buffer removed. The beads were then washed twice with 500μl 1X coupling buffer. The antibody was then diluted 1:20 with 20X coupling buffer and IP lysis/wash buffer to a final concentration of 5μg in 100μl. This was then added to the beads and incubated with rotation for 15 minutes at room temperature. The beads were then collected and the antibody solution removed. The beads were then washed three times once with 100μl 1X coupling buffer and then twice with 300μl 1X coupling buffer.

2.5.3.3 Crosslinking the Bound Antibody

Briefly, crosslinking reagent was prepared combining 2.5µl 20X coupling buffer, 4µl 0.25mM Disuccinimidyl suberate (DSS, a protein cross-linking agent, which forms stable amide bonds with the amine side chains of the antibody) and 43.5µl ultrapure water. This was then added to the beads and incubated with rotation for 30 minutes at room temperature. The beads were then collected using a magnetic stand and the crosslinking reagent removed. The beads were then washed three times with 100µl elution buffer for five minutes, then twice with 200µl ice cold IP lysis/wash buffer.

2.5.3.4 Immunoprecipitation of the Protein

The protein was diluted to a concentration of 300µg per 500µl and this was added to the crosslinked magnetic beads and incubated overnight at 4°C with rotation. The beads were then collected and the supernatant removed. The beads were then washed twice with 500µl of IP lysis/wash buffer and once with 500µl ultrapure water. Finally the protein was eluted off the beads with 50µl elution buffer. Then 5µl neutralization buffer was added to the eluate in order to neutralize the low pH. The resulting samples were then diluted 1:5 with reducing loading buffer and then boiled for 5 minutes at 100°C. A fifth of this was loaded per lane of the Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE) gels.

2.5.4 Optimisation of Block

Two of the antibodies used are raised in goat (KCNMA1 channel N-terminus, sc-14746; CACNA1C channel C-terminus, sc-16230) and require an anti-goat secondary antibody to be used. However anti-goat secondary antibodies can bind to bovine IgG as well as goat IgG (Buchwalow, Samoilova *et al.* 2011). If this were to happen and the membrane had been blocked with a block containing bovine IgG it could result in the secondary antibody binding to the block and giving very high background. Routinely either dried milk or BSA is used as a block, BSA contains bovine IgG and commercial milk often contains some bovine IgG contamination. To test if there is any interaction between the block and the secondary antibody, nitrocellulose membrane was blocked for two hours at room temperature. The membrane was then washed using TBS-T as follows:

2 x quick rinses

1 x Fifteen minute wash
2 x Five minute washes

After washing the membranes were incubated in a 1:20,000 or 1:40,000 dilution of HRP conjugated secondary antibody for one hour at room temperature. The membranes were the washed a final time and then developed using ECL reagents according to the manufacturers guidelines using the Syngene G:BOX Chemi-16 gel documentation system followed by visualisation with Syngenes Ingenius Bioimaging software GeneSnap and GeneTools Version 4 quantification software (SynGene, Cambridge UK)

2.5.5 Dot Blot

Dot blots are a quick and effective method of determining the optimum, protein concentration and primary antibody dilution to use during SDS-PAGE and Western Blotting.

A range of protein concentrations (5, 10 and 50µg) was spotted onto nitrocellulose membrane and allowed to dry. The membrane was then blocked using either TBS-TM, or Non-protein block (Pierce) for one hour at room temperature. The membrane was then washed using TBS-T as follows:

2 x quick rinses
1 x Fifteen minute wash
2 x Five minute washes

Then each blot was incubated with primary antibody, overnight at 4ºC with gentle agitation. The following day the membranes were washed as detailed previously and then incubated in a 1:10,000 dilution of HRP conjugated secondary antibody for one hour at room temperature. The membranes were the washed a final time and then developed using ECL reagents according to the manufacturers guidelines using the Syngene G:BOX Chemi-16 gel documentation system followed by visualisation with Syngenes Ingenius Bioimaging software GeneSnap and GeneTools Version 4 quantification software (SynGene, Cambridge UK)

2.5.6 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were analysed by SDS-PAGE. Samples were diluted either 1:1 with SDS-loading buffer (prepared to a final volume of 25.0ml: 3.0ml 500mM Tris pH 6.8, 2.4 ml glycerol, 4.8 ml 10% (w/v) SDS, 1.2 ml β-mercaptoethanol, 600 µl 0.05% (w/v) bromophenol blue and 12.0 ml MQ water) or 1:4 with Lane Marker Reducing Sample Buffer (Thermo Scientific) depending on the concentration of the protein sample. Samples were then boiled at 95ºC for ten minutes before briefly centrifuging for 30 seconds at 1,200*g* in a Sigma 1-14 microcentrifuge.

For the large L- and CACNA1G calcium channel proteins (~170kDa and ~262kDa respectively) a gradient system was employed using 4 – 20% precast gels (Bio-Rad). The gel was subject to electrophoresis at 150V until the dye had migrated to the end of the gel cassette. A discontinuous 8% gel was used for the smaller KCNMA1 channel (~100kDa) and C-Terminal of the CACNA1C Channel again the gel was subject to electrophoresis at 150V until the dye had migrated to the end of the gel cassette.

2.5.7 Wet Transfer

The larger proteins from the 4-20% precast gel underwent a wet transfer. Briefly, a nitrocellulose membrane and six pieces of 3mm Whatman paper were cut to the size of the gel and soaked in Tris Glycine buffer (TGB: 25mM Tris, 192mM Glycine, 0.1% (w/v) SDS and 10% (v/v) Methanol) Three pre-soaked pieces of Whatman paper and the nitrocellulose membrane were stacked onto the foam on the black side of the cassette the gel was rinsed in TGB and layered on top of the nitrocellulose membrane followed by three Whatman papers, foam and the red side of the cassette. The cassette was closed and place into the Geneflow omniPAGE electroblotting tank and the tank filled with TGB. The proteins were transferred for 2.5 hours at 4ºC and 40V. The membrane was stained using PonceauS solution to determine if the transfer was successful.

2.5.8 Semi-Dry Transfer

The smaller proteins from the 8% gel underwent a semi-dry transfer. Briefly, a nitrocellulose membrane and six pieces of 3mm Whatman paper were cut to the size of the gel and soaked in Towbin's Transfer buffer (TTB: 25mM Tris pH 7.4, 192mM Glycine and 20% (v/v) Methanol) Three pre-soaked pieces of Whatman paper were

stacked onto the platinum anode of the Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) and rolled with a glass rod to remove any air bubbles. The nitrocellulose membrane was then placed onto the Whatman paper and rolled again to remove air bubbles. The gel was rinsed in TTB and layered on top followed by three Whatman papers and rolled for a final time to remove any air bubbles. The cathode was placed onto the stack and the latches engaged. The safety cover was then placed on the unit and the proteins were transferred for 20 minutes at 20V. The membrane was stained using Ponceau-S solution to determine if the transfer was successful.

2.5.9 Western Blot

The membrane was rinsed in TBS and blocked in TBS-TM at room temperature for two hours. The blot was washed in TBS-T for two quick rinses, one 15 minute wash and two five minute washes. The primary antibody was incubated either overnight at 4ºC (APC-021) or overnight at room temperature (ACC-003, ACC-021, ab104624 and sc-16230). The blot was washed in TBS-T for two quick rinses, one 15 minute wash and two five minute washes. An HRP-conjugated secondary antibody was incubated with the blot for one hour at room temperature, before washing in TBS-T for two quick rinses, one 15 minute wash and two five minute washes. The blot was developed using ECL reagents according to the manufacturers guidelines using the Syngene G:BOX Chemi-16 gel documentation system followed by visualisation with Syngenes Ingenius Bioimaging software GeneSnap and GeneTools Version 4 quantification software (SynGene, Cambridge UK). After development of the blot, manual band quantification was performed using the Gene Tools software from Syngene (Geneflow, Staffs). All experiments were performed a minimum of three times and results are expressed as the mean ± SEM. All data analyses were conducted on GraphPad Prism Version 6 (GraphPad Software, San Diego, California) where a one way ANOVA with Dunett's post-test was performed to compare the individual stimulations against the un-stimulated control; $p<0.05$ was considered statistically significant.

2.6 RNA Methods

2.6.1 Total RNA Extraction

Cells were cultured in 25cm^2 flasks. On reaching 90% confluence the cells were stimulated 10ng/ml TNF or, 2µg/ml TSA or, 10ng/ml TNF followed by 2µg/ml TSA or, 2µg/ml TSA followed by 10ng/ml TNF with a non-stimulated 25cm^2 flask serving as a control. The cells were then processed by discarding the medium, washing the cells briefly with sterile PBS containing 1mM EDTA in order to remove any traces of serum prior to the addition of trypsin (6U/ml), 3mls of trypsin was added and the flask incubated at 37ºC for five minutes. The detached cells were then transferred to a 15ml centrifuge tube and centrifuged at 250*g* for five minutes. Total RNA was extracted from the cell pellet using the EZRNA extraction Kit (Geneflow Lichfield, Staffs. U.K.), Briefly, the medium was removed and the cells lysed directly in the flask by addition of 0.5ml denaturing solution, the lysed cells were scraped off the flask and transferred to a clean microcentrifuge tube, and incubated at room temperature for five minutes. An equal volume (0.5 ml) of extraction solution was then added, the samples mixed vigorously, then incubated at room temperature for 15 minutes and then centrifuged at 12,000*g* for 15 minutes at 4ºC. The aqueous colourless (upper) phase was transferred to a fresh microcentrifuge tube and total RNA precipitated by the addition of an equal volume of isopropanol (0.5 ml) and subsequent incubation at -20ºC overnight. Following this the sample was centrifuged at 16,000*g* for 15 minutes at 4ºC and the supernatant discarded. The pellet was washed by vortexing in 1ml 75% (v/v) ethanol and then centrifuged at 16,000*g* for five minutes at 4ºC, the supernatant was discarded. The pellet was allowed to air dry for five-to-ten minutes and then dissolved in 25µl Diethylpyrocarbonate (DEPC,) water. DEPC water was used as it inactivates RNase enzymes by the covalent modification of the active site of histidine, lysine, cysteine and tyrosine residues. The extracted total RNA was quantified using the nanophotometer by measuring UV absorbtion at 260nm, using the Beer Lambert Law where an absorbtion of one equates to a concentration of 50µg/ml RNA. The ratio of UV absorbtion at A260 and A280nm was used to assess the purity of the sample. The $A_{260/280}$ for pure RNA is ~2.

2.6.2 cDNA Synthesis of Cultured Myocyte mRNA

cDNA synthesis was performed using Bio-Rad iScript cDNA synthesis Kit according to the manufacturer's guidelines, briefly 1µg of total RNA was added to 4µl of 5x iScript reaction mix and made up to 20µl with nuclease free water. The reaction was then incubated as follows:

5 minutes at 25ºC
30 minutes at 42ºC
5 minutes at 85ºC

Hold at 4ºC

One microlitre of cDNA was used per PCR.

2.7 The Chromatin Immunoprecipitation (ChIP) Assay

Two confluent 75cm^2 flasks of myocytes were stimulated with 10ng/ml TNF for one hour, two confluent 75cm^2 flasks of myocytes were stimulated with 2µg/ml TSA for 24hrs, with two non-stimulated 75cm^2 flasks serving as controls. The ChIP assay was carried out using the Millipore EZ magna ChIP Kit according to the manufacturer's guidelines.

2.7.1 *In Vivo* Crosslinking and Lysis

Briefly, the cells were fixed by the addition of 37% formaldehyde to the media to a final concentration of 1% for ten minutes. Glycine was added to a final concentration of 125mM for five minutes to quench any unreacted formaldehyde. The medium was removed and the cells washed twice in ice cold PBS.

After the second wash the cells were scraped off the flask into 2ml of ice cold PBS containing protease inhibitors. The cells were centrifuged at 800*g* for five minutes at 4ºC. The cell pellet was resuspended in 0.5mls cell lysis buffer containing protease inhibitors and incubated on ice for 15 minutes with brief vortexing every five minutes. The cell suspension was centrifuged at 800*g* for five minutes at 4ºC. The cell pellet was resuspended in 0.5ml nuclear lysis buffer.

2.7.2 Sonication to Shear DNA

The cell suspension was then sonicated using a vibra cell 150 (sonics) with an exponential probe at maximum amplitude for ten seconds three times with ten second intervals and then centrifuged at 12,300*g* for ten minutes at 4ºC to remove any insoluble material. The sheared chromatin was the aliquoted into 50µl aliquots with one being retained to analyse sheared chromatin size by agarose gel electrophoresis.

2.7.3 Immunoprecipitation of Crosslinked Protein/DNA

Each aliquot was diluted one in ten with ChIP dilution buffer containing protease inhibitors. An input sample representing the starting material was taken. Protein G magnetic beads were prepared by pre-absorbing them with salmon sperm DNA to reduce background, 20µl of these prepared beads was added to each aliquot along with 5µg of either SP1, MZF, CBP, RelA, p50 or KCNMA1 C terminal antibody or a rabbit IgG negative control and left to incubate overnight, on a roller at 4ºC. Protein G beads were captured with a magnetic separator and the complex washed for five minutes each with a low salt buffer, a high salt buffer, a lithium chloride buffer and a TE buffer.

2.7.4 Elution of Protein/DNA complexes and Reversal of Crosslinks of Protein/DNA complexes to free DNA

The chromatin:RelA antibody:magnetic bead complex was resuspended in 100µl ChIP elution buffer containing 1µl Proteinase K and incubated at 62ºC for two hours with shaking in a Stuart Orbital Incubator S1500 followed by a ten minute incubation at 95ºC. The magnetic beads were then captured using a magnetic separator and the supernatant containing the chromatin was removed to a fresh tube.

2.7.5 DNA Purification Using Spin Columns

The chromatin was then purified using Sigma Genelute PCR Clean-up Kit columns (Sigma) according to the manufacturer's protocol. Briefly, 500µl of column preparation solution was added to each column, centrifuged and the flow through discarded. The DNA solution was diluted in five volumes of DNA binding solution, mixed, added to the column then centrifuged for one minute and the flow through discarded. The filter was

washed with 500µl ethanol wash solution, centrifuged and the flow through discarded. The column was then re-centrifuged to remove any residual ethanol. DNA was eluted from the filter by applying 30µl of ultrapure water and incubating at room temperature for one minute before centrifuging. This was then repeated with 20µl ultrapure water to maximise DNA yield. All centrifugations were performed at 16,000*g*.

2.8 The Methylated DNA Immunoprecipitation (MeDIP) Assay

Two confluent 75cm^2 flasks of myocytes were stimulated with 10ng/ml TNF for one hour, two confluent 75cm^2 flasks of myocytes were stimulated with 2µg/ml TSA for 24hrs, with two non-stimulated 75cm^2 flasks serving as controls. The MeDIP assay was carried out using the EpiQuik Methylated DNA Immunoprecipitation Kit (Epigentek Farmingdale NY USA) according to the manufacturer's guidelines.

2.8.1 Antibody Binding to Assay Plates

Briefly, each well was washed once with 150µl CP1 wash buffer, then 100µl CP2 antibody buffer was added to each well followed by 1µl of either Anti-5-methylcytosine, or normal mouse IgG. The wells were sealed and incubated at room temperature for one hour.

2.8.2 Cell Collection

The culture medium was removed from the tissue culture flasks and the cells washed once with PBS. Then 500µl CP3B lysis buffer was added to each flask and incubated at room temperature for five minutes. Finally the cell suspension was pipetted up and down several times to break the cells.

2.8.3 DNA Shearing

The cell solution was then transferred to a microcentrifuge tube and incubated at room temperature for five minutes. The cell solution was then vortexed vigorously for ten seconds. The cell suspension was then sonicated using a vibra cell 150 (sonics) with an exponential probe at maximum amplitude for ten seconds three times with ten second intervals. The sonicate was then clarified by centrifugation at 12,300*g* for ten minutes at 4ºC to remove any insoluble material.

2.8.4 Methylated DNA Immunoprecipitation

One hundred and sixty microliters of the supernatant from above was then transferred to a new microcentrifuge tube and 160µl CP4 ChIP dilution buffer added. To serve as the input 7.5µl was removed and placed into a new microcentrifuge tube. The antibody solution was then removed from the wells and the wells washed three times with 150µl CP2 antibody buffer. Then 150µl of the cell lysate was added to each well. The wells were sealed and incubated at room temperature for 90 minutes on a rocking platform at 50-100rpm. The supernatant was then removed and the wells washed six times with 150µl CP1 wash buffer for two minutes each at 100rpm on a rocking platform (Stuart). Followed by a wash with 150µl TE for two minutes at 100rpm on a rocking platform.

2.8.5 Manual DNA Isolation / purification

One microliter Proteinase K was added to 40µl CP5 DNA release buffer and mixed. This was then added to each well including the input wells, a further 40µl CP6 reverse buffer was added to the input wells. The wells were then sealed and incubated at 65°C for 15 minutes, then 40µl CP6 reverse buffer was added to each of the sample wells and the wells re-sealed and incubated at 65°C for 30 minutes. For each sample a spin column was added to a 2ml collection tube, then 150µl CP7 binding buffer was added to each sample and the sample transferred to a spin column. The spin columns were centrifuged at 10,500*g* for 20 seconds, then 200µl 70% (v/v) ethanol was added to each column and the columns spun at 10,500*g* for 20 seconds. Next 200µl 90% (v/v) ethanol was added to each column and the columns spun again at 10,500*g* for 20 seconds, then 200µl 70% (v/v) ethanol was added to each column and the columns spun at 10,500*g* for 20 seconds. The spin column was then placed in a new collection tube and 20µl CP8 elution buffer was added to each column which was then centrifuged at 10,500*g* for 20 seconds. The DNA was then stored at -20°C

2.9 Polymerase Chain Reaction (PCR)

2.9.1 Primer Design

For characterisation of the primary cultures Actin α2 (NM_001141945.1) was chosen as an indicator of the presence myocytes and Thy-1 cell surface antigen (NM_006288.3), as an indicator of the presence of fibroblasts. Primers were designed to amplify each of the RNAs (Table 2.1), PHM1 RNA was used as a positive control for actin α2 and a negative control for Thy-1, fibroblast RNA was used as a positive control for Thy-1. Optimised PCR conditions are detailed in Table 2.2.

Table 2.1 Primers

Region of Interest		Primer Sequence
actin α2	Forward	5'-TGGCTTGGCTTGTCAGGGCTTG-3'
	Reverse	5'-CGGGTGCTCAGAACGCTGGA-3'
Thy-1	Forward	5'-CTGGGTGCAGCAACCGGAGG-3'
	Reverse	5'-TGCTCAGGCACCCCCACAGT-3'

Table 2.2 PCR Conditions

Region of Interest	Annealing Temperature	Duration of Denature/Anneal/elongation	No. of Cycles	Product size (bp) and
Actin α2	64.6	25 / 30 / 45	30	239
Thy-1	65	25 / 30 / 45	25	307

For the detection of channel splice variants within the primary cultures a combination of a literature review and Genbank sequence data was used to design primers to cover an un-spliced region in each channel and also to cover a number of spliced regions in each channel. Details of the primers are listed in Table 2.3.

For the ChIP and MeDIP PCRs the TransFac promoter database (http://www.gene-regulation.com/pub/databases.html) was used to identify the channel promoters. Then the MethPrimer database (http://www.urogene.org/methprimer/) was used to identify CpG islands within these promoter regions and also to design primers to cover the promoter regions of the three channels. Details of the primers are listed in Table 2.4 also included are the IκBα primers which were used as a positive control for the RelA ChIP.

Table 2.3 KCNMA1, CACNA1C and CACNA1G channel and splice variant primers

Channel / Region	Primer	Sequence (5'-3')
KCNMA1	Forward	5'- CGGAGGCAGCAGTCTTAG-3'
	Reverse	5'-AAGAAAGTCACCATGGAGGAG-3'
KCNMA1 132 (M1)	Forward	5'-CTCCTCCATGGTGACTTTCTT-3'
	Reverse	5'-TTACAAGTGCACCGATGCTG-3'
KCNMA1 2 (M2)	Forward	5'-GGAAACCGCAAGAAATAC-3'
	Reverse	5'-ACCTCATGGAGAAGAGGTTG-3'
KCNMA1 srkr (M3)	Forward	5'-GGTCTGTCCCACNA1GCTACTGT-3'
	Reverse	5'-CAAAGATGCAGACCACGACA-3'
KCNMA1 strex (M4)	Forward	5'-GTGCCAGCAACTTTCATTAC-3'
	Reverse	5'-TCAGGGTCATCATCATCGTC-3'
KCNMA1 5 (M5)	Forward	5'-ACAGCATTTGCCGTCAGTG-3'
	Reverse	5'-AATATTCAAGGCAGACAAAG-3'
CACNA1G	Forward	5'-AAGTGCTACAGCGTGGAGGC-3'
	Reverse	5'-CTCTGACCCAGCAGACCTGG-3'
T 14 (T2)	Forward	5'-GTTTGCCTCTGAGCGGGATG-3'
	Reverse	5'-AGGCTGGGTGAGAAGAAATC-3'
T 25a (T4)	Forward	5'-GGGCGAGGATACCAGGAACA-3'
	Reverse	5'-GCTGCCGACACTTGTGGAAG-3'
T 25C (T6)	Forward	5'-GGAGAAGCGCCTACGAAGAC-3'
	Reverse	5'-CCGATGACACCTGTGATGAA-3'
T 30 (T8)	Forward	5'-CATGCGGGCGCTGCTGGACA-3'
	Reverse	5'-GAGACTCGGAAGAGGGTTAG-3'
CACNA1C	Forward	5'-GCCCTATGTGGCCCTCCTGATCGTGAT-3'
	Reverse	5'-CTTGTCCAGCTCCTCCTCAGCGGTGAGA-3'
L 8 / 8* (L4)	Forward	5'-CAGTGCCAGAACGGCACGGT-3'
	Reverse	5'-CGCTCAACACACCGAGAACCA-3'
	Reverse	5'-CGCTAAGCACACCGAGAACCA-3'
L 31 (L10)	Forward	5'-GGAATACGCCCTCAAGGCCCG-3'
	Reverse	5'-GGGAGAGCATTGGGTATGTTCAGC-3'
L 41 (L11)	Forward	5'-TGGTCCATCCTTGGTCCCCACC-3'
	Reverse	5'-AGCAGCGGACACAGCCTCCT-3'

Table 2.4 KCNMA1, CACNA1C and CACNA1G channel promoter primers and ChIP positive control primers

Channel / Region	Primer	Sequence (5'-3')	Product Size (bp)
KCNMA1 5' CpG	Forward	5'-GGCGTGGCGACAGGACTAGG-3'	407
	Reverse	5'-CCCGAGCGCCGAGAGCCAG-3'	
KCNMA1 3' CpG	Forward	5'-CCTGGCTCTCGGCGCTCGG -3'	509
	Reverse	5'-TTGGGCTCGTGGACCGAGGA -3'	
CACNA1C CpG 1	Forward	5'-GCGCGAGGGGGTGTG-3'	532
	Reverse	5'-TCTCTGCTGTCAAAGGGAGC-3'	
CACNA1C CpG 2	Forward	5'-TTGCCGGCTCCCTTTGACAGC-3'	343
	Reverse	5'-GCCGTGGTCTGCAGCGTTTC-3'	
CACNA1G CpG1	Forward	5'-CCGCCCCTCCCGGACAGTGA-3'	475
	Reverse	5'-GGGCTCTAGGGCGCAAGCTGA-3'	
CACNA1G CpG2	Forward	5'-CGCCCTAGAGCCCACCAGAT-3'	377
	Reverse	5'-CGTGCCCCGAAGGATATGGGTT-3'	
IκBα	Forward	5'-GACGACCCCAATTCAAATCG-3'	300
	Reverse	5'-TCAGGCTCGGGGAATCACNA1G-3'	

2.9.2 PCR Reactions

For the cell characterisation and splice variant PCRs a *Taq* polymerase from Promega was used. The 25µl reaction volume was comprised of 12.5µl PCR Master Mix (Promega), 50pmol of both forward and reverse primers, 2µl cDNA and 9.5µl ultrapure water. The individual PCR reaction conditions are detailed in Table 2.5.

For the ChIP and MeDIP PCRs, Agilent's Herculase II Fusion Polymerase was used as it is optimised for the amplification of GC-rich regions. The 25µl reaction was comprised of 5µl Herculase buffer, 0-4% (v/v) DMSO, 125µM dNTPs, 0.125µM both forward and reverse primers, 0.25µl Herculase II fusion DNA Polymerase (note that the manufacturer, Agilent, did not provide a value for the number of enzyme units/µl), 1-2µl DNA and then up to 25µl with ultrapure water. These conditions were optimised for each of the individual primer pairs. The optimised conditions are listed in Table 2.6.

Table 2.5 PCR Conditions for KCNMA1, CACNA1C and CACNA1G channel and splice variant PCRs

Channel / Region	Annealing Temperature	Duration of Denature/Anneal/elongation	No. of Cycles	Product size (bp) and exon complement
KCNMA1	52	20/45/60	35	242
KCNMA1 132 (M 1)	54	25/30/45	35	(+ 132) 437/ (Δ132) 305
KCNMA1 2 (M 2)	50	25/30/45	35	565
KCNMA1 srkr (M 3)	54	25/30/45	35	547
KCNMA1 strex (M 4)	52	25/30/45	35	(+ strex) 622/ (Δ strex) 535
KCNMA1 5 (M 5)	53	25/30/45	35	857
CACNA1G	63	30/30/60	40	394
T 14 (T2)	55.4	25/30/45	40	(+14) 360/ (Δ14)291
T 25a (T4)	57	25/30/45	40	(+25A) 311/ (Δ25A) 209
T 25C (T6)	55.4	25/30/45	40	(+25C+26) 219 / (Δ25C+26) 198/ (+25CΔ26) 165 / (Δ25CΔ26) 144
T 30 (T8)	58	25/30/45	40	(+30B+31A) 213 / (Δ30B+31A) 192 / (+30BΔ31A) 164 / (Δ30BΔ31A) 143
CACNA1C	63	30/60/30	40	940
L 8 / 8* (L4)	59	25/30/45	40	244
L 31 (L10)	59	25/30/45	40	(+30+31+32+33+34) 454 / (+30+31+33+34) 370 / (+30+32+33+34) 343 / (+30+32+34) 259
L 41 (L11)	59	25/30/45	40	(+40+41^{+}+42+43) 672 / (+40^{-}+40b+43^{+}) 643 / (+40+41+42+43) 615

Table 2.6 PCR Conditions for KCNMA1, CACNA1C and CACNA1G ChIP PCRs

Channel / Region	Annealing Temperature	Duration of Denature/Anneal/elongation	No. of Cycles
KCNMA1 5' CpG	54.6	20/30/20	34
KCNMA1 3' CpG	60.3	20/30/20	34
CACNA1C CpG 1	54.9	20/30/30	38
CACNA1C CpG 2	54.5	20/30/20	38
CACNA1G CpG1	61.6	20/30/30	38
CACNA1G CpG2	58.8	20/30/30	34
IκBα	60.0	45/60/60	38

2.9.3 Agarose Gel Electrophoresis

The PCR products were analysed by agarose gel electrophoresis. A 1.5% (w/v) agarose gel was used for the analysis of the characterisation PCRs and a 3.0% (w/v) agarose gel for the channel splice variant PCRs. The gels were prepared by adding either 1.5g (1.5% w/v) or 3.0g (3% w/v) agarose to 100ml Tris Acetate EDTA (TAE; 40mM Tris Acetate and 1mM EDTA) buffer. This was heated in a microwave on high power until the agarose had completely dissolved. The liquid gel was then cooled to around 55ºC and GelRed solution, a DNA intercalating agent which fluoresces under ultra violet light and enables the visualisation of double stranded DNA, added at a 10,000x dilution (Cambridge Bioscience). The gel was then poured into a pre-prepared gel cast and left to set at room temperature. The gel was placed into a gel tank and covered in one times TAE buffer. DNA loading dye (0.03% bromophenol blue, 0.03% xylene cyanol FF, 0.4% orange G, 15% Ficoll®400, 10mM Tris-HCL [pH 7.5] and 50mM EDTA [pH 8.0] Promega) was added to each sample at a six times dilution and loaded into the gel. A current of 80V was applied across the gel for around 40 minutes and the separated DNA fragments were visualised using UV light at a wavelength of 310nm. Syngene G:BOX Chemi-16 gel documentation system followed by visualisation with Syngenes Ingenius Bioimaging software GeneSnap and GeneTools Version 4 quantification software (SynGene, Cambridge UK).

2.9.4 Analysis of PCR Products

Bands were quantified using manual band quantification software in GeneTools. Briefly, a box was drawn around the band taking care not to include any non-specific banding, this box was copied across all visible bands by double clicking the mouse, to ensure the box remained the same size.

The percentage of myocytes and fibroblasts in the primary cultures was calculated using the following equations:

$$\text{Percentage Myocytes} = \left(\frac{(\text{Mean pixel value actin } \alpha 2 - \text{Mean pixel value Thy-1})}{\text{Mean Pixel value actin } \alpha 2} \right) \text{X } 100$$

$$\text{Percentage Fibroblasts} = \left(\frac{\text{Mean pixel value Thy-1}}{\text{Mean Pixel value actin } \alpha 2} \right) \text{X } 100$$

The analysis of the splice variant PCRs was performed as follows: first each individual sample set and PCR reaction were analysed separately. Within each sample set and PCR reaction the un-stimulated sample was quantified as 100% the stimulated samples for the matching PCR reactions were then quantified as a percentage of the un-stimulated reaction. The data for each sample set was then loaded into Prism and a one way ANOVA for matched samples with Dunette's post-test was performed to compare the individual stimulations against the un-stimulated control.

2.9.5 Extraction of DNA from an Agarose Gel

The DNA bands were extracted from the gel for sequence analysis using Geneflow Q-Spin Extraction Purification Kit. Briefly, the DNA band was excised from the gel and transferred to a clean microcentrifuge tube. An equal volume (w/v) binding buffer was added to the gel slice and incubated at 65ºC for ten minutes with occasional vortexing. The dissolved gel was then added to a spin column incubated at room temperature for two minutes and then centrifuged at 6,000*g* for one minute. The flow through was discarded and 500µl wash solution added to the column, and the column centrifuged at 6,000*g* for

15 seconds. The column was then centrifuged for a further minute to remove any residual wash solution and then placed into a clean collection tube. Thirty micolitres elution solution was added to the column and incubated for two minutes at room temperature and then centrifuged at 10,500*g* for one minute. The eluted DNA was sent for sequencing at the Medical School Genomics Core Facility.

2.10 ChIP Sequencing Methods

2.10.1 Preparation of Samples

2.10.1.1 ChIP DNA Whole Genome Amplification (WGA)

Primary myocyte ChIP chromatin from 2.7.1, including both the KCNMA1 and IgG fractions were amplified using the Sigma Whole Genome Amplification Kit (WGA) as detailed in the manufacturer's instructions with slight modifications. One microlitre of ChIP DNA was diluted with 9µl of ultrapure water. Two microlitres of library preparation buffer together with 1µl of library stabilisation solution was added to this and heated at 95ºC for two minutes before cooling on ice. One microlitre of library preparation enzyme was then added and the reaction incubated in the thermal cycler for the following:

20 minutes at 16ºC (pre-cooled to this temperature)
20 minutes at 20ºC
20 minutes at 37ºC
5 minutes at 75ºC
4ºC hold

During the incubation a master mix containing 7.5µl of 10x amplification master mix, 47.5µl ultrapure water and 5µl (2 units/µl) of Whole Genome Amplification (WGA) DNA polymerase was prepared. This was added to each sample and incubated in the thermal cycler for the following:

3 minutes at 95ºC
Then 20 cycles of:
15 seconds at 95ºC
5 minutes at 65ºC

4ºC hold

Ten percent of the amplified DNA was run on an agarose gel to ensure a DNA smear of the correct size was obtained. The remainder of the DNA was purified using Sigma Genelute™ PCR Clean-Up Kit following the manufacturers guidelines, as detailed in section 2.7.5.

2.10.1.2 Re-Amplification of Amplified DNA

One round of amplification did not produce enough DNA for ChIP sequencing experiments, therefore, the amplified DNA was re-amplified using the WGA re-amplification kit to generate the 7.5µg required. The method was as follows: 1µl of amplified DNA was added to 9µl of ultrapure water. As master mix containing 47.5µl ultrapure water, 7.5µl amplification master mix, 2.5units WGA polymerase and dNTPS (10 mM dGTP, 10 mM dCTP, 10 mM dTTP and 10 mM dATP) was prepared. This was added to each sample and incubated in the thermal cycler for the following:

3 minutes at 95ºC
Then 20 cycles of:
15 seconds at 95ºC
5 minutes at 65ºC
4ºC hold

Ten percent of the amplified DNA was run on an agarose gel to ensure a DNA smear of the correct size was obtained. The remainder of the DNA was purified using Sigma Genelute™ PCR Clean-Up Kit following the manufacturer's guidelines, as detailed in section 2.7.5. The DNA was quantified using the nanophotometer by measuring UV absorbtion at 260nm. Using the Beer Lambert Law where an absorbtion of one equates to a concentration of 50µg/ml DNA. The ratio of UV absorbtion at A260 and A280nm was used to assess the purity of the sample. The $A_{260/280}$ for pure DNA is ~1.8-2.0.

2.10.1.3 ChIP Sequencing End Repair

The ChIP sequencing (ChIP-seq) was carried out by Dr Paul Heath in Sheffield Institute for Translational Neuroscience (SITrAN). End Repair was carried out using the Kapa

Biosystems Library Preparation Kit according to the manufacturer's instructions. Briefly, a reaction mix comprising 8µl water, 7µl 10x Kapa end repair buffer, 5µl Kapa end repair enzyme mix and 50µl fragmented DNA was prepared and incubated at 20°C for 30 minutes. Following this 120µl of Agencourt® AMPure® XP Reagent was added to each reaction and incubated at room temperature for 15 minutes. This reaction was then placed on a magnet until the supernatant was clear. The supernatant was discarded and the beads washed twice with 200µl 80% (v/v) ethanol.

2.10.1.4 Addition of 'A' Bases to the 3' end of the DNA Fragments

This was again carried out using the Kapa Biosystems Library Preparation Kit according to the manufacturer's instructions. Briefly, a reaction mix comprising the beads (from above), 42µl water, 5µl 10x KAPA A tailing buffer, 3µl KAPA A tailing enzyme was prepared. This was incubated at 30°C for 30 minutes. This reaction was then placed on a magnet until the supernatant was clear. The supernatant was discarded and the beads washed twice with 200µl 80% (v/v) ethanol.

2.10.1.5 Ligate Adapters to DNA Fragments

At this point the protocol was switched to the NEBNext Ultra DNA Library Preparation Kit and the manufacturer's protocols followed. Briefly, a reaction mix comprising the beads (from above), 15µl Blunt/TA Ligase Master Mix, 2.5µl NEBNext Adaptor for Illumina, 1µl Ligation Enhancer was prepared. This mix was incubated at 20°C for 15 minutes. At this point 3µl USER enzyme was added to the mix and incubated at 37°C for 15 minutes. This reaction was then placed on a magnet until the supernatant was clear. The supernatant was discarded and the beads washed twice with 200µl 80% (v/v) ethanol. The DNA was then eluted from the beads by the addition of 22µl 10mM Tris HCl and incubation at room temperature for two minutes. This reaction was then placed on a magnet until the supernatant was clear. The supernatant was collected.

2.10.1.6 Enrichment of the Adapter-Modified DNA Fragments by PCR.

The following PCR reaction mix was prepared: 20µl DNA (from above), 2.5µl Index Primer/i7 Primer, 2.5µl Universal primer/i5 primer, 25µl NEBNext High Fidelity 2x PCR Master Mix. This was then amplified using the following PCR protocol:

30 seconds at 98°C
Then 18 cycles of:
10 seconds at 98°C
30 seconds at 65°C
30 seconds at 72°C
Then:
5 minutes at 72°C
Hold at 4°C

The DNA was then again purified and eluted in 28µl. The library was then validated using the bioanalyser. The bioanalyser utilises chip based electrophoresis to analyse RNA and DNA, a dye is intercalated into the sample which then runs past a filter the fragments are detected by laser induced fluorescence. Purity, size and concentration of the sample is then calculated by calibration against the ladder which is of known size and concentration. Briefly, 1µl of both the construct and the negative control are loaded onto the bioanalyser and the size purity and concentration of the sample were checked.

2.10.1.7 Cluster Generation by Bridge Amplification

The flow cell surface is coated with single stranded oligonucleotides corresponding to the adaptor sequences which were ligated to the DNA. These single stranded adapter ligated fragments were bound to the surface of the flow cell in the presence of reagents for polymerase based extension. The flow cell and bound DNA then underwent a series of denaturation and extension cycles resulting in localised amplification of single molecules in millions of unique locations across the flow cell surface.

2.10.1.8 Sequencing by Synthesis

The flow cell (from above) which now contains millions of unique clusters was then loaded into the sequencer for automated cycles of extension and imaging.

2.10.2 Analysis of ChIP Sequencing Data

The .fastq files (raw data) were imported into Galaxy (https://usegalaxy.org), an open access web based platform for Next Generation Sequencing (NGS) analysis. Galaxy is a

framework for integrating computational tools, the majority of NGS analysis tools can be run from the Galaxy interface. Initially, for quality control, summary statistics were computed for each sample. This was done by running the "NGS: QC and Manipulation > FASTQ Summary Statistics" tool. Next the "NGS: QC and Manipulation > FASTQ Groomer" tool was used to convert the FASTQ data to Sanger data. The next step was to map the reads onto a reference genome, the H19 build of the human genome was loaded as the reference genome and the "NGS: Mapping > Map with Bowtie for Illumina" tool was used to carry out the mapping. Once the reads were mapped peak calling was performed using the "NGS: Peak Calling > SICER" tool. This is a statistical tool for the identification of ChIP enriched regions. Once significantly enrich regions were located the NCBI Map Viewer (http://www.ncbi.nlm.nih.gov/mapview/) was used to identify genes associated with the enriched regions.

2.10.3 ChIP Sequencing Motif Enrichment Analysis

To perform the motif enrichment analysis the first step was to retrieve the genomic DNA sequences for the Peaks located above. This was done using the "NGS: Fetch Sequence < extract genomic DNA" tool. This generated a FASTA file containing the genomic DNA sequences. To identify overexpressed motifs the Panoptic Motif Search Tool (http://www.pms.engr.uconn.edu/index.php?page=motifseq) was used. Panoptic Motif Search is a motif discovery algorithm which has been designed to find short, core DNA-binding motifs for Eukaryotic transcription factors and is optimised to handle large ChIP-seq datasets. The FASTA data generated in Galaxy was loaded into the Panoptic Motif Search tool and the motif discovery algorithm run.

2.10.4 ChIP PCR Validation of ChIP Sequencing

For validation of ChIP-seq peaks three promoters that where shown to be bound by the KCNMA1 protein were selected; MAP3K7, JHP2 and RB1. Primers were designed to amplify a portion of the promoter sequence for each of these genes which was identified though ChIP- seq peak calling (Table 2.7). Optimised PCR conditions are detailed in Table 2.8. PCR was carried out as detailed in section 2.9.2 utilising Agilents Herculase II Fusion Polymerase.

Table 2.7 ChIP Validation Primers

Gene	Primer	Sequence (5'-3')	Product Size (bp)
MAP3K7	Forward	5'-TGATGGCGCTTTGAGACCC-3'	127
	Reverse	5'- ATGCATCTGCCACCATCAGAA -3'	
JHP2	Forward	5'- CTGTGCTGGAAGCAAGCATAC -3'	141
	Reverse	5'- AGGGTGCTGAAGAACACCG -3'	
RB1	Forward	5'- TGCCAGGTATGAAGGAACTGTG -3'	117
	Reverse	5'- ACTACGTGCAAAGATAAGACCA -3'	

Table 2.8 ChIPseq Validation Optimised PCR Conditions

Gene	Annealing Temperature	Duration of Denature/Anneal/elongation	No. of Cycles
MAP3K7	54.4	20/30/15	30
JHP2	55.3	20/30/15	30
RB1	54.4	20/30/15	30

Chapter 3: The KCNMA1, CACNA1C and CACNA1G Channels Expression in the Cultured Myometrial Cells Results and Discussion*

*This chapter of the thesis has been published in part in - Waite, S. L., S. V. Gandhi, R. N. Khan and N. R. Chapman (2014). "The effect of trichostatin-A and tumor necrosis factor on expression of splice variants of the KCNMA1 and CACNA1C channels in human myometrium." Front. Physiol. **5**: 261. (Waite, Gandhi *et al.* 2014)

3.1 Introduction

As detailed in Chapter One *Introduction*, in the human myometrium, the cessation of uterine quiescence and the onset of both normal and preterm labour are associated with a number of pro-inflammatory cytokines, including, but not limited to, IL-1β, TNF and IL-8. It has been previously demonstrated that external agents such as TNF (potent pro-inflammatory cytokine) can induce myometrial contractility while other compounds, namely trichostatin-A (TSA), can promote myometrial relaxation in isolated human smooth muscle strips (Lu, Mazet *et al.* 1999, Moynihan, Hehir *et al.* 2008, Webster, Waite *et al.* 2013). The exact means by which this process occurred could not be elucidated. It is likely, however, that both agents could influence both ion channel expression and subsequent RNA splicing, which, in turn, would modulate myometrial contractility. Given the effects previously reported for both TNF and TSA on primary human myometrium and myometrial myocytes (Webster, Waite *et al.* 2013), this chapter aims to examine the expression and splicing pattern of the KCNMA1 channel, the CACNA1C and the CACNA1G in cultured human myometrial cells and define whether these parameters were influenced by TNF and TSA.

TNF is a potent pro-inflammatory cytokine which is secreted in response to inflammatory stimuli. TNF stimulates the inflammatory cascade through NFκB, which in turn promotes the expression of pro-contractile genes (Mendelson 2009). Within the myometrium activation of NFκB by TNF has been shown to promote the expression of a number of labour associated genes (Chan, van den Berg *et al.* 2014, Cookson, Waite *et al.* 2015). Therefore, it is possible that the TNF may increase the contractility of the myometrium via an effect on the expression or splicing pattern of the KCNMA1, the CACNA1C and the CACNA1G channels.

TSA is a broad spectrum lysine de-acetylase inhibitor, and so prevents the removal of the acetyl groups from the histones and other proteins. Prevention of the removal of these acetyl groups from histone lysines will have the net effect of maintaining the more open accessible state of the DNA, this may be a mechanism by which it affects the expression or splicing of the channels. However, it is important to remember that TSA also prevents the removal of lysine acetyl groups from other proteins and the relaxatory effect of TSA

may also be as a result of this general increase in lysine acetylation. Chen *et al.* demonstrated that TSA could promote the relaxation of arterial tone in vascular smooth muscle cells via the increased acetylation of several myofilamentous proteins (Chen, Karolczak-Bayatti *et al.* 2013).

3.2 Characterisation of Myometrial Cell Cultures

Full details of the methods used in this chapter can be found in Chapter 2 *Materials and Methods*.

3.2.1 Identification of Cells in the Myometrial Cell Cultures

The aim of this chapter was to determine if TNF and TSA exerted their effect on myometrial smooth muscle contractility via the regulation of the expression of either the KCNMA1, CACNA1C and CACNA1G or their splice variants. As a first step the myometrial cells derived from the biopsies were characterised to verify their suitability as samples for this purpose. To this end it was first established that the myometrial cell cultures consisted primarily of myocyte cells rather than fibroblasts. This was done by measuring the expression of Actin α2, a smooth muscle actin and Thy-1 a cell surface antigen expressed specifically from fibroblasts. An immortalised human myometrial cell line, PHM1-31 (Monga, Ku *et al.* 1996) was used as a smooth muscle positive control and oral fibroblasts as a fibroblast cell positive control. PCR using Thy-1, showed only faint bands (Figure 3.1A) in the PHM1-31 cell line and the myometrial cell cultures indicting there is only a low level of fibroblast or myofibroblast contamination. In contrast to this and as expected, the fibroblast culture showed a much more intense Thy-1 band (Figure 3.1A). All cultures gave an intense band when Actin α2 primers were employed in the PCR indicating the presence of smooth muscle cells (Figure 3.1B). Importantly, within the myometrial cell cultures the level of fibroblast contamination was found to vary between 1.2 and 11% which is comparable with the immortalised PHM1-31 cell line (Figure 3.1C).

3.2.2 Channel Immunocytochemistry

Culturing of primary cells has been shown to be able to affect certain functional properties of the cells such as the loss of steroid receptors (Berns, Brinkmann *et al.* 1985, Tyagi,

Tyagi *et al.* 2006). The effect of culturing on ion channel expression is unclear. Consequently it was important to determine if the myocyte cell cultures expressed the KCNMA1, CACNA1C and CACNA1G channels.

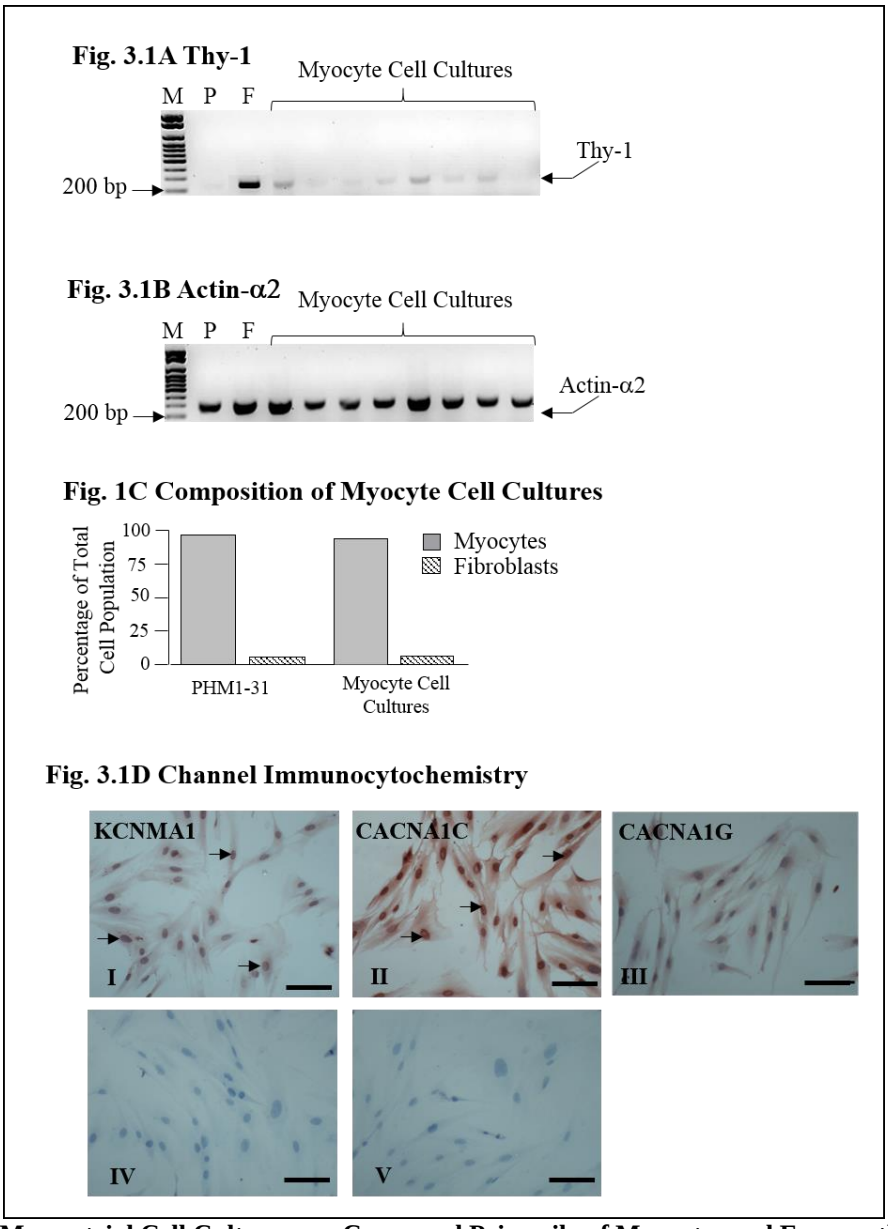


Figure 3.1 Myometrial Cell Cultures are Composed Primarily of Myocytes and Express the KCNMA1, CACNA1C and CACNA1G Channels

RNA from myometrial cell cultures was extracted and amplified by PCR. **(A)** Myometrial cell cultures express a low level of Thy-1 mRNA indicating a low level of Fibroblasts in the culture. **(B)** Myometrial cell cultures express a high level of Actin-α2 mRNA indicating a high level of myocytes in the culture. Manual quantification of the relative band intensities was used to confirm the presence of myocytes and to estimate the level of fibroblast contamination. **(C)** Myometrial cell cultures are comprised of 88 – 98% myocytes (grey bar) and between 1.2 and 11% fibroblasts (striped bar) which is comparable to the PHM1 cultures. **(D)** Myometrial cells were staining using antibodies specific to KCNMA1 (**I**) and CACNA1C (**II**) CACNA1G (**III**) channels. Controls excluded primary (**IV**) and secondary antibody (**V**). Dark red/brown staining denotes specific staining of the protein of interest (scale bar is 100µm).

Immunocytochemistry demonstrated that all three channel types remain present in myometrial cell cultures (KCNMA1 - Figure 3.1D, Panel I; CACNA1C – Figure 3.1D, Panel II; CACNA1G – Figure 3.1D, Panel III Antiserum controls Figure 3.1D, Panels IV-V). Interestingly, intense nuclear staining (Figure 3.1D Panels I-II black arrows) was observed for both the KCNMA1 and CACNA1C channels although the significance of this remains unclear at present.

3.2.3 Myocyte Cell Culture Contractility

The final step in characterising the myocyte cell cultures was determining if they retained the ability to contract. A collagen contraction assay was used for this purpose. Myocyte cells were embedded within collagen gels and either left un-stimulated or stimulated with TNF (Webster, Waite *et al.* 2013) or TSA (Chen, Karolczak-Bayatti *et al.* 2013, Webster, Waite *et al.* 2013) or Oxytocin, as a contraction-positive control (Mitchell, Fang *et al.* 1998, Mukaddam-Daher, Yin *et al.* 2001, Robinson, Schumann *et al.* 2003). PHMI-31 cells and HEK293 cells left un-stimulated or stimulated with TNF or TSA or Oxytocin were used as positive and negative controls respectively. The area of the gels was recorded to determine what effects the stimulants had on the contractility of the cells. A reduction in the surface area of the gel after the gel was released was an indication that the cells retained smooth muscle tone, i.e. the resistance to passive stretch during resting state. Upon the release of the gels from the sides of the well there was a visible reduction in the size of the gels containing both cultured myometrial cells and PHM1-31 cells, there was no reduction in gel size seen for the HEK293 cells. This demonstrates that the cultured myometrial cells still retain contractile tone. The amount of contraction was calculated as the inverse of the gel size after release expressed as a percentage of the original gel size (Figure 3.2A).

In the collagen gels containing HEK293 cells, no contraction was observed upon treatment with Oxytocin or TNF (Figure 3.2B). In contrast to this, however, treatment with Oxytocin caused contraction in both collagen embedded PHM1-31 and primary cells demonstrating that both these cells can be induced to contract (Figure 3.2B). Also, when collagen embedded PHM1-31 or primary cells where stimulated with TNF, there was a significant reduction in gel surface area afterwards suggesting that TNF was inducing cell contraction (Figures 3.2B, 3.2C).

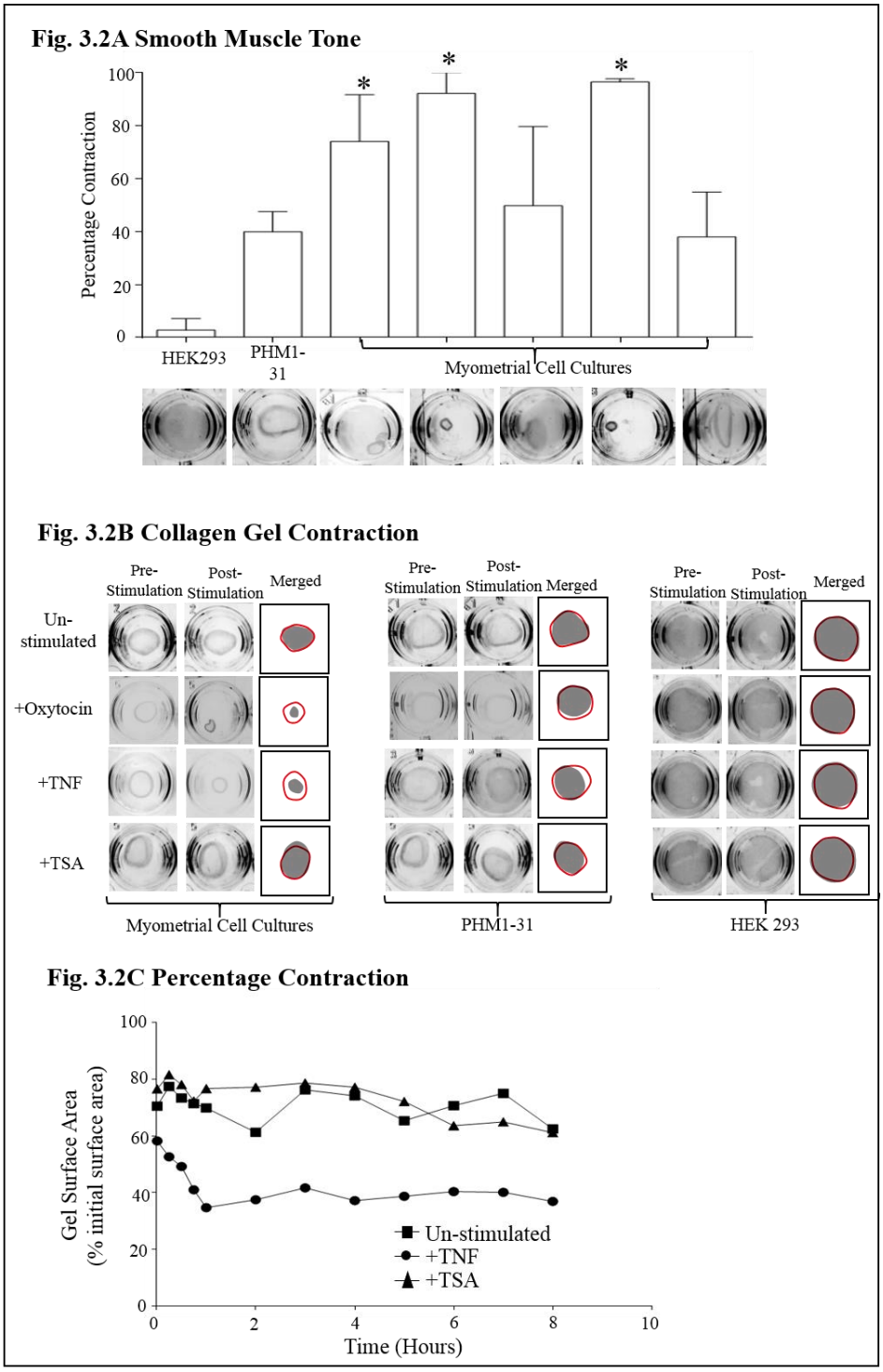


Figure 3.2 Myometrial Cell Cultures Retain Smooth Muscle Tone and the Ability to Contract and Relax.

(A) The reduction of the surface area of the collagen after release demonstrates the retention of smooth muscle tone in the myometrial cell cultures. In the more confluent cultures this reduction in gel size becomes significant ($p<0.05$ *) There is no reduction in gel size in the HEK293 cultures. 10ng/ml TNF, 100ng/ml TSA, 10ng/ml Oxytocin were added to the cells within the collagen gels with non-stimulated cells serving as a control. The cells were then imaged at specific time points between 5 minutes and 8 hours post-stimulation. **(B)** Depiction of the maximum gel contraction for each culture after stimulation**. (C)** Depiction of the change in collagen gel surface area of the primary cells over the course of the experiment (n=5).

Significantly, an increase in gel surface area was observed when cultures were treated with TSA indicating that it induced a loss of basal tone in the collagen-embedded myocytes cultures although this did not reach statistical significance (Figures 3.2B, 3.2C).

3.3 KCNMA1, CACNA1C and CACNA1G Channel Expression and Splice Variant Expression

Full details of the methods used in this chapter can be found in Chapter 2 *Materials and Methods*.

For the detection of channel splice variants within the primary cultures, primers were selected to cover an un-spliced region in each channel and also to cover a number of spliced regions in each channel. The individual PCR primer sequences are detailed in Chapter 2 *Materials and Methods* Table 2.3; individual splice variant-specific PCR conditions are listed in Chapter 2 *Materials and Methods* Table 2.5. All channel RT-PCR reactions were done simultaneously and with the GAPDH control serving experiments for both KCNMA1, CACNA1C and CACNA1G channels. Consequently Figure 3.3D is duplicated in Figures. 3.4D-3.8D purely for this reason.

3.3.1 KCNMA1 Splice Variants are Expressed in Myometrial Cell Cultures and are Down Regulated by Trichostatin A

Previous studies, have reported differential effects of both TNF and TSA on signalling pathways in myometrial cells (Chapman, Smyrnias *et al.* 2005, Webster, Waite *et al.* 2013). This present research sought to determine if such compounds could also influence expression of the KCNMA1 mRNA. Total cellular RNA was amplified using primers specific for a region within KCNMA1 that is conserved in all splice variants. As such, this would give an indication of overall expression of the mRNA for this channel and how TNF and TSA affected this. Figure 3.3A demonstrates that TSA was seen to induce a significant reduction in expression of KCNMA1 mRNA (Figure 3.3A white bar). In contrast to this, TNF was seen to induce small but statistically significant increase in the expression of the KCNMA1 channel mRNA (Figure 3.3A grey bar). Neither treatment influenced the expression of GAPDH (Figure 3.3D).

The KCNMA1 channel is documented to have a number of splice variants (Curley, Morrison *et al.* 2004). Consequently, it was important to determine if the expression of such variants also occurred in human myometrial cell cultures. Moreover, it was also salient to determine which of these variants were sensitive to TNF or TSA treatment. Total cellular RNA was amplified using primers specific for the different splice sites published for the KCNMA1 open reading frame. Myometrial cell cultures were seen to express a number of different splice variants M1 (including both the Mk44 variants which either express a 132bp insert between exons 1 and 2 or have this insert omitted); M2 (+exon 11 and 12); M3 (+exon 19); M4 (STREX-2) and M5 (+exon 29) (Figure 3.3B, C).

When cultures were subsequently exposed to either TNF or TSA, TSA significantly decreased the expression of the Mk44 splice variants in comparison to un-stimulated cultures and the ratio between the Mk44 variant containing the 132bp insert and the variant lacking this insert was also significantly reduced with TSA stimulation. TNF had no effect on either the expression of the Mk44 variant or on the ratio between the insert and insert-less forms of the variant (Figure 3B). We also observed a similar TSA-induced repression of both the M3 (+ exon 19 variant) and the M4 (STREX-2 variant) (Figure 3.3B).

The observed reduction in STREX-2 levels, while statistically significant, was, however, small in magnitude and the relevance of this remains unclear at present. Representative gels of each treatment are illustrated in Figure 3.3C. No change in GAPDH was observed with either treatment (Figure 3.3D).

Fig. 3.3A Total KCNMA1 Channel Expression

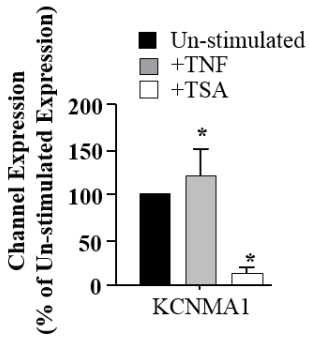


Fig. 3.3B KCNMA1 Channel Splice Variant Expression - Quantitation

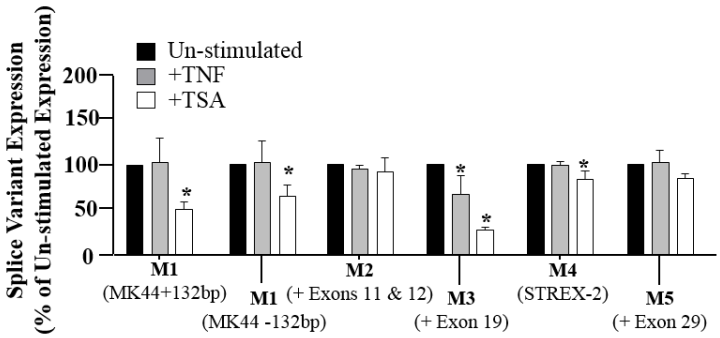


Fig. 3.3C KCNMA1 Channel Splice Variant Expression – Representative Gels

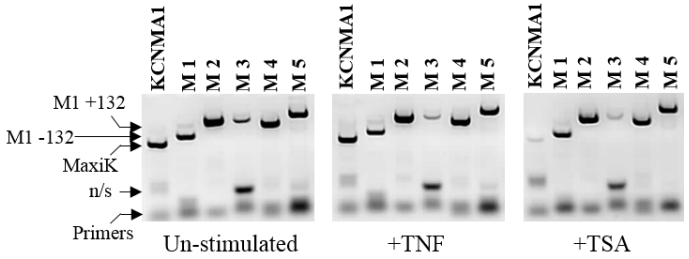


Fig. 3.3D GAPDH Expression

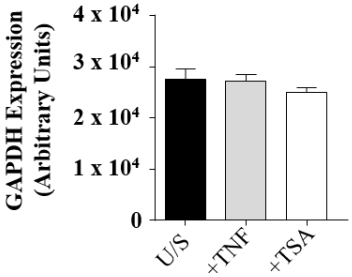


Figure 3.3 The Expression of the KCNMA1 Channel and Splice Variants in Myometrial Cell Cultures
Myometrial myocyte cell cultures were stimulated with 10ng/ml TNF for 1 hour, 100ng/ml TSA for 24 hours or left un-stimulated. Total RNA was extracted, reverse transcribed and amplified using channel and splice variant specific primers. Manual quantification of the relative band intensities were used to quantify the level of expression and this was expressed as a percentage of the un-stimulated expression (n = 8). **(A)** TNF significantly increased and TSA significantly reduced the expression of the KCNMA1 channel ($p<0.05$ *). **(B)** Myometrial cell cultures express a range of KCNMA1 splice variants, TNF had no effect on splice variant expression and TSA significantly reduced the expression of the Mk44 splice variant ($p<0.05$ *). **(C)** Representative gel of the RT-PCR products. (**D)** Neither TNF nor TSA influenced the expression of GAPDH.

3.3.2 The Combined Effect of TNF or TSA Stimulus on the Expression of the KCNMA1 Channel and its Splice Variants in Myometrial Cell Cultures are Dependent on the Order in Which they Occur.

Next it was determined if these compounds had an enhancing or diminishing effect on each other. When the cultures were exposed to TNF prior to exposure to TSA the expression of the KCNMA1 channel (Figure 3.4A, C) and the M3 splice variant (Figure 3.4B, C) were significantly reduced in comparison to un-stimulated cultures. In contrast, the STREX-2 and M5 variants were significantly up regulated (Figure 3.4B, C) in comparison to un-stimulated cultures. Indicating that when TNF is present prior to TSA stimulation the effect of these two compounds varies according to which splice variant is examined.

In terms of the KCNMA1 channel and the M3 variant this effect enhanced the effect of TSA, whereas, with the STREX-2 and M5 variants the effect was different to that seen for either of the individual compounds. The relevance of this remains unclear at present.

When the cultures were exposed to TSA prior to exposure to TNF the Mk44 variant containing the 132bp insert, and the M3 variant were significantly down regulated (Figure 3.4B, C) in comparison to un-stimulated cultures. Although the repression of these two variants was also observed with TSA stimulation, the addition of TNF following TSA had the effect of reducing the level of repression to a small degree. This indicates that when TNF exposure follows TSA exposure it has a small diminishing effect on the action of TSA. No change in GAPDH was observed with either treatment (Figure 3.4D).

Fig. 3.4A Total KCNMA1 Channel Expression

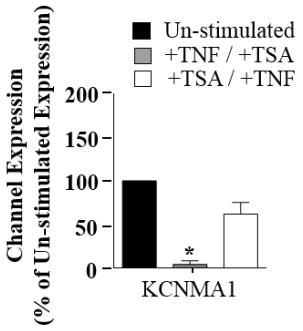


Fig. 3.4B KCNMA1 Channel Splice Variant Expression - Quantitation

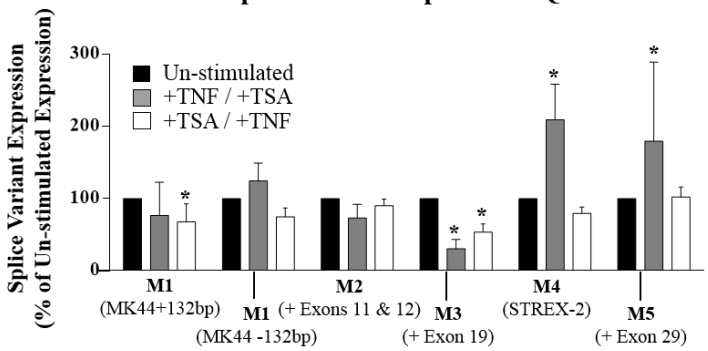


Fig. 3.4C KCNMA1 Channel Splice Variant Expression – Representative Gels

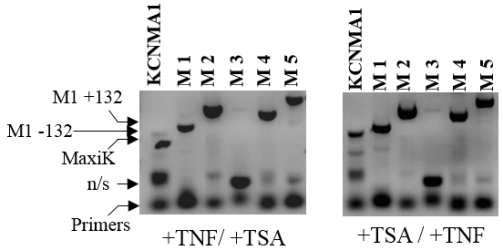


Fig. 3.4D GAPDH Expression

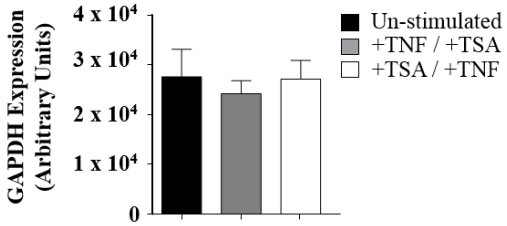


Figure 3.4 The Expression of the KCNMA1 Channel and Splice Variants in Myometrial Cell Cultures
Myometrial myocyte cell cultures were stimulated with 10ng/ml TNF for 1 hour followed by 100ng/ml TSA for twenty-four hours, 100ng/ml TSA for twenty-four hours followed by 10ng/ml TNF for 1 hour or left un-stimulated. Total RNA was extracted, reverse transcribed and amplified using channel and splice variant specific primers. Manual quantification of the relative band intensities were used to quantify the level of expression and this was expressed as a percentage of the un-stimulated expression (n = 5). **(A)** TSA/TNF had no significant effect whilst TNF/TSA significantly reduced the expression of the KCNMA1 channel ($p<0.05$ *). **(B)** Myometrial cell cultures express a range of KCNMA1 splice variants, TNF/TSA had no effect on splice variant expression with the exception of M3 which was significantly reduced ($p<0.05$ *) and TSA/TNF significantly reduced the expression of the MK44+132bp and M3 (+19) splice variants ($p<0.05$ *). TSA/TNF also significantly increased the expression of the M4 (STREX-2) and M5 (+29) splice variants ($p<0.05$ *) **(C)** Representative gel of the RT-PCR products. (**D)** Neither TNF/TSA nor TSA/TNF influenced the expression of GAPDH.

3.3.3 CACNA1C Splice Variants are Expressed in Myometrial Cell Cultures and are Down Regulated by Trichostatin A

Next, it was determined if these compounds also influence expression of the CACNA1C mRNA. Total cellular RNA was amplified using primers specific for a region within CACNA1C that is conserved in all splice variants. As such, this would give an indication of overall expression of the mRNA for this channel and how TNF and TSA affected this. Figure 3.5A demonstrates that TSA was seen to induce a reduction in expression of CACNA1C mRNA but this did not reach significance (Figure 3.5A white bar). In contrast to this, TNF was seen to induce a significant increase in total CACNA1C mRNA expression (Figure 3.5A grey bar). Neither treatment influenced the expression of GAPDH (Figure 3.5D).

The CACNA1C channel is documented to have a number of splice variants (Tang, Liang *et al.* 2004). Consequently, it was important to determine if the expression of such variants also occurred in myometrial cell cultures. Moreover, it was also salient to determine which of these variants were sensitive to TNF treatment. Total cellular RNA was amplified using primers specific for the different splice sites published for the CACNA1C open reading frame (Tang, Liang *et al.* 2004). Myometrial cell cultures were seen to express a number of different splice variants (Figures 3.5B, 3.5C). Briefly, the myocytes express the splice site 4 variant containing exon 8* this variant has lower DHP sensitivity, more rapid activation and slower deactivation kinetics than the alternative exon 8-containing variant (Soldatov 1992).

Two splice site 10 variants were observed one containing exons 30, 31, 32, 33 and 34 and a second containing exons 30, 31, 33 and 34 these two variants affect the size and rigidity of the S3 to S4 linker segments. It has been suggested that shorter linker segments result in channels with slower gating kinetics while longer linking segments result in channels with faster gating kinetics (Perez-Reyes, Wei *et al.* 1990, Yang, Chen *et al.* 2000). Of the two variants found in the myometrial cells the one containing 30, 31, 33 and 34 is the shorter variant and was found in approximately 80% of the RNA. Finally the splice site 11 variant containing exons 40, 41, 42 and 43 was also observed.

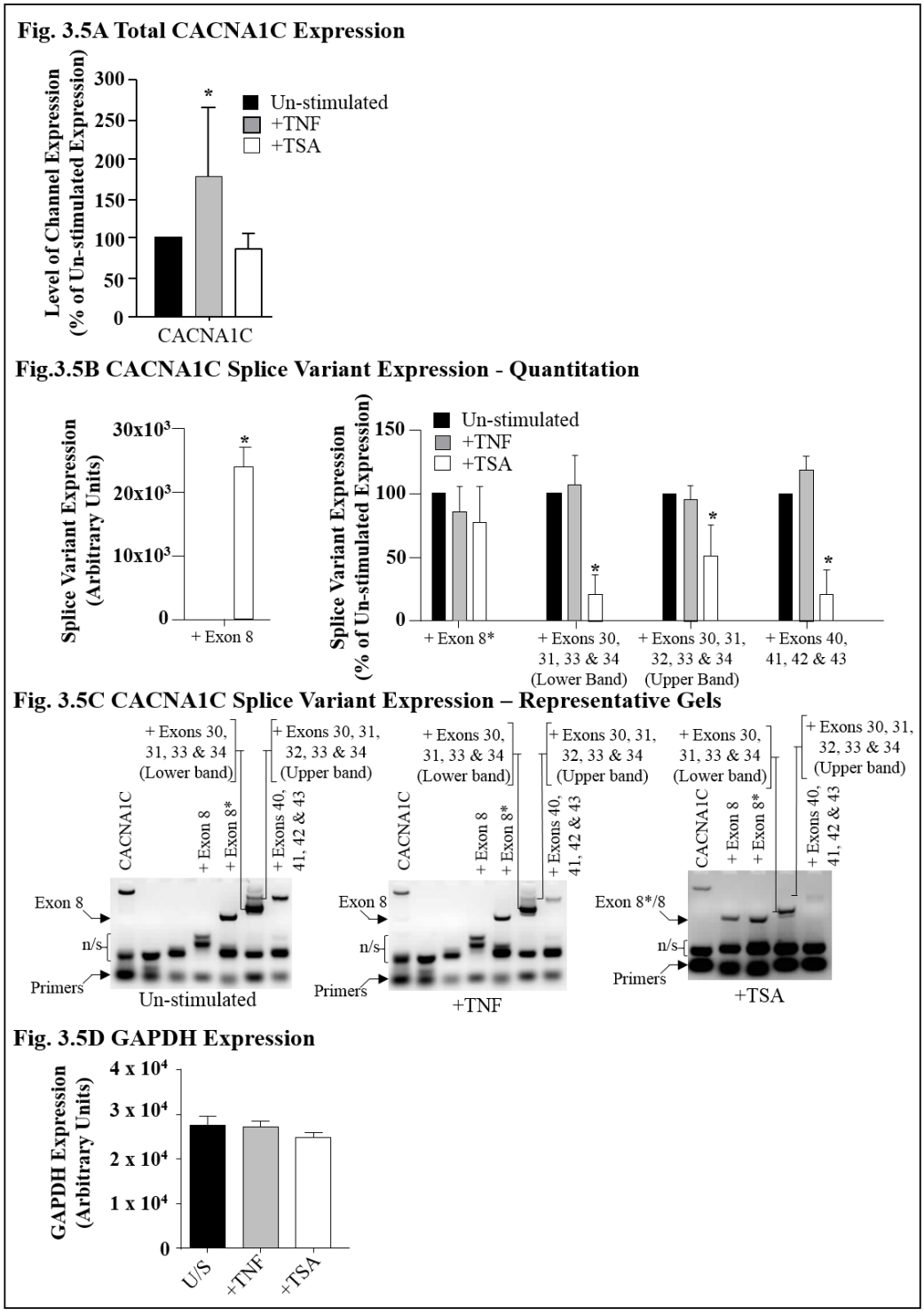


Figure 3.5 The Expression of the CACNA1C Channel and Splice Variants in Myometrial Cell Cultures

Myometrial myocyte cell cultures were stimulated with 10ng/ml TNF for 1 hour, 100ng/ml TSA for 24 hours or left un-stimulated. Total RNA was extracted, reverse transcribed and amplified using channel and splice variant specific primers. Manual quantification of the relative band intensities were used to quantify the level of expression and this was expressed as a percentage of the un-stimulated expression (n = 7). **(A)** TNF significantly increased the expression of the CACNA1C channel whilst TSA had no significant effect ($p<0.05$ *). **(B)** Myometrial cell cultures express a range of CACNA1C splice variants. TSA significantly induced expression of the exon-8 variant (Left Panel; $p<0.05$ *) whilst also reducing expression of a number of other splice variants (Right Panel; $p<0.05$ *). TNF had no effect on splice variant expression. **(C)** Representative gel of the RT-PCR products. **(D)** Neither TNF nor TSA influenced the expression of GAPDH.

When cultures were subsequently exposed to either TNF or TSA, TSA resulted in the novel expression of CACNA1C splice variants utilising exon 8 (Figure 3.5B; Left Panel). Un-stimulated cells and those treated with TNF exclusively utilise exon 8*; after treatment with TSA, however, approximately 45% of the mRNA expressed contained the exon 8 variant. The utilisation of exon 8 leads to the expression of channels with higher DHP sensitivity and slower activation and more rapid deactivation curves (Figures 3.5B, 3.5C).

TSA also resulted in the down regulation of both forms of the splice site 10 variant while TNF had little effect (Figures 3.5B, 3.5C). The effect of TSA is what would be expected as the faster gating kinetics of the longer form would mean that it would take less time for the channels to fully open and hence would increase the influx of calcium and would promote contraction. The effect of a decreased expression of this variant would therefore lead to relaxation (Figures 3.5B, 3.5C).

TSA also resulted in a down regulation of the splice site 11 variant. All the treatment groups expressed the same variant at splice site 11, this variant contained exons 40, 41, 42 and 43 with no additions or deletions. After TSA treatment the expression of this variant was significantly reduced, there was, however, no alternative variant expressed (Figures 3.5B, 3.5C). Neither treatment influenced the expression of GAPDH (Figure 3.5D).

3.3.4 The Combined Effect of TNF or TSA Stimulus on the Expression of the CACNA1C Channel and its Splice Variants in Myometrial Cell Cultures are Dependent on the Order in Which they Occur.

Next, as with the KCNMA1 channel it was determined if these compounds had an enhancing or diminishing effect on each other. When the cultures were exposed to TNF prior to exposure to TSA the expression of the CACNA1C (Figure 3.6A, C) and the splice site 11 variant (Figure 3.6B, C) were both significantly down regulated in comparison to un-stimulated cultures. The splice site 4 variant containing exon 8 (Figure 3.6B left panel, C) was also expressed. When administered in this order the presence of TNF enhances

the effect of TSA on both the CACNA1C and the splice site 11 variant. The presence of TNF had no effect on the expression of the splice site 4 variant containing exon 8.

When the cultures were exposed to TSA prior to exposure to TNF the expression of the CACNA1C (Figure 3.6A, C), both splice site 10 variants and the splice site 11 variant (Figure 3.6B, C) were all significantly down regulated in comparison to un-stimulated cultures. The presence of TNF enhanced TSA repression of the CACNA1C to significant level. However, the presence of TNF did not alter the effect of TSA alone on either of the splice site 10 variants or the splice site 11 variant. The presence of TNF, again, had no effect on the expression of the splice site 4 variant containing exon 8 (Figure 3.6B left panel, C). No change in GAPDH was observed with either treatment (Figure 3.6D).

3.3.5 CACNA1G Splice Variants are Expressed in Myometrial Cell Cultures and are Up Regulated by TNF

Next, it was determined if these compounds also influence expression of the CACNA1G mRNA. Total cellular RNA was amplified using primers specific for a region within CACNA1G that is conserved in all splice variants. As such, this would give an indication of overall expression of the mRNA for this channel and how TNF and TSA affected this. Figure 3.7A demonstrates that TSA was seen to induce an increase in expression of CACNA1G mRNA but this did not reach significance (Figure 3.7A white bar). Neither treatment influenced the expression of GAPDH (Figure 3.7D).

The CACNA1G is documented to have a number of splice variants (Chemin, Monteil *et al.* 2001, Bertolesi, Walia Da Silva *et al.* 2006, Emerick, Stein *et al.* 2006, Shcheglovitov, Vitko *et al.* 2008, David, Garcia *et al.* 2010). Consequently, it was important to determine if the expression of such variants also occurred in human myometrial cells. Moreover, it was also salient to determine which of these variants were sensitive to TNF treatment. Total cellular RNA was amplified using primers specific for the different splice sites published for the CACNA1G open reading frame (Emerick, Stein *et al.* 2006). myometrial cell cultures were seen to express a number of different splice variants (Figures 3.7B, 3.7C).

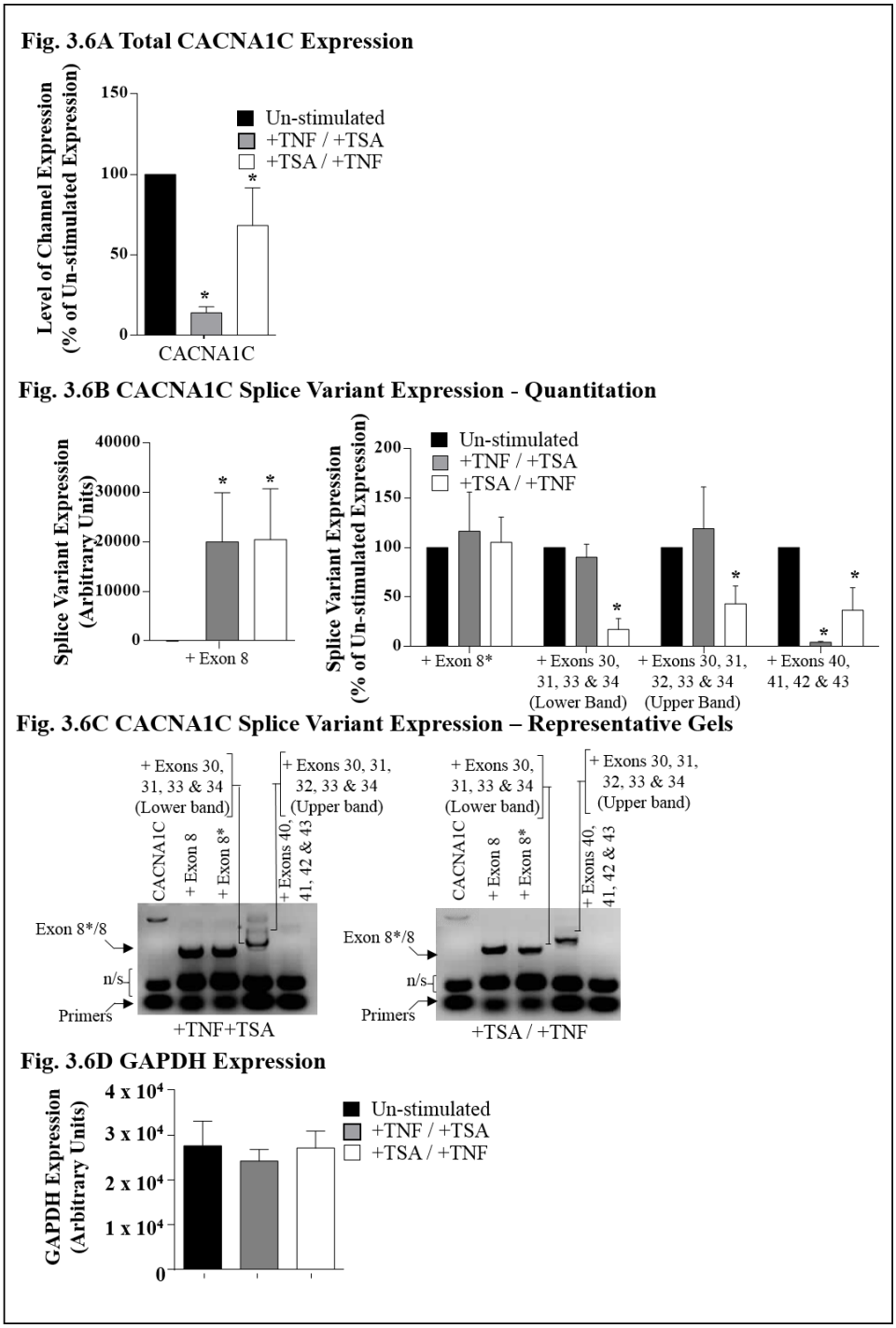


Figure 3.6 The Expression of the CACNA1C Channel and Splice Variants in Myometrial Cell Cultures

Myometrial myocyte cell cultures were stimulated with 10ng/ml TNF for 1 hour followed by 100ng/ml TSA for twenty-four hours, 100ng/ml TSA for twenty-four hours followed by 10ng/ml TNF for 1 hour or left un-stimulated. Total RNAwas extracted, reverse transcribed and amplified using channel and splice variant specific primers. Manual quantification of the relative band intensities were used to quantify the level of expression and this was expressed as a percentage of the un-stimulated expression (n = 4). **(A)** Both TNF/TSA and TSA/TNF significantly reduced the expression of the CACNA1C channel ($p<0.05$ *). **(B)** Myometrial cell cultures express a range of CACNA1C splice variants. Both TNF/TSA and TSA/TNF significantly induced expression of the exon-8 variant (Left Panel; $p<0.05$ *). TSA/TNF also reduced expression of a number of other splice variants (Right Panel; $p<0.05$ *). TNF/TSA had no effect on splice variant expression with the exception of splice site 11 variant containing exons 40, 41, 42 and 43 which was significantly reduced (Right Panel $p<0.05$ *). **(C)** Representative gel of the RT-PCR products. **(D)** Neither TNF/TSA nor TSA/TNF influenced the expression of GAPDH.

Briefly, within the myocytes a splice site 2 variant excluding exon 14 was observed, this variant will lack a PKC phosphorylation site (Bertolesi, Walia Da Silva *et al.* 2006). At splice site 4 the variant observed contained exon 25 without the utilisation of the splice acceptor sites and so would form a functional channel (Chemin, Monteil *et al.* 2001, Bertolesi, Walia Da Silva *et al.* 2006, Emerick, Stein *et al.* 2006). The splice site 5 variant observed excluded exon 25C and contained exon 26, this will modify the channels gating properties (Chemin, Monteil *et al.* 2001, Bertolesi, Walia Da Silva *et al.* 2006, Emerick, Stein *et al.* 2006). Two variants were observed at splice site 7/8 one containing both exon 30B and 31A, and one excluding exon 30B and containing exon 31A. Exon 30B modifies the voltage dependence of the channel (Emerick, Stein *et al.* 2006), however, exon 31A results in prematurely truncated protein (Emerick, Stein *et al.* 2006), and so both these variants will result in truncated protein.

When cultures were subsequently exposed to either TNF or TSA, TNF resulted in a small but significant up regulation of splice site 2 variant excluding exon 14 (Figure 3.7B, C), this variant will lack a PKC phosphorylation site (Bertolesi, Walia Da Silva *et al.* 2006). PKC has been demonstrated to stimulate CACNA1G activity and so the loss of a PKC site would reduce the stimulatory effect of PKC on the channel (Park, Kang *et al.* 2006).

TNF also resulted in the significant up-regulation of the splice site 4 variant containing exon 25, this forms a functional channel which will activate and inactivate at more hyperpolarised potentials (Hawkins and Kearney 2012). This will have the effect of allowing these channel to open at more negative membrane potentials and so will support contraction.

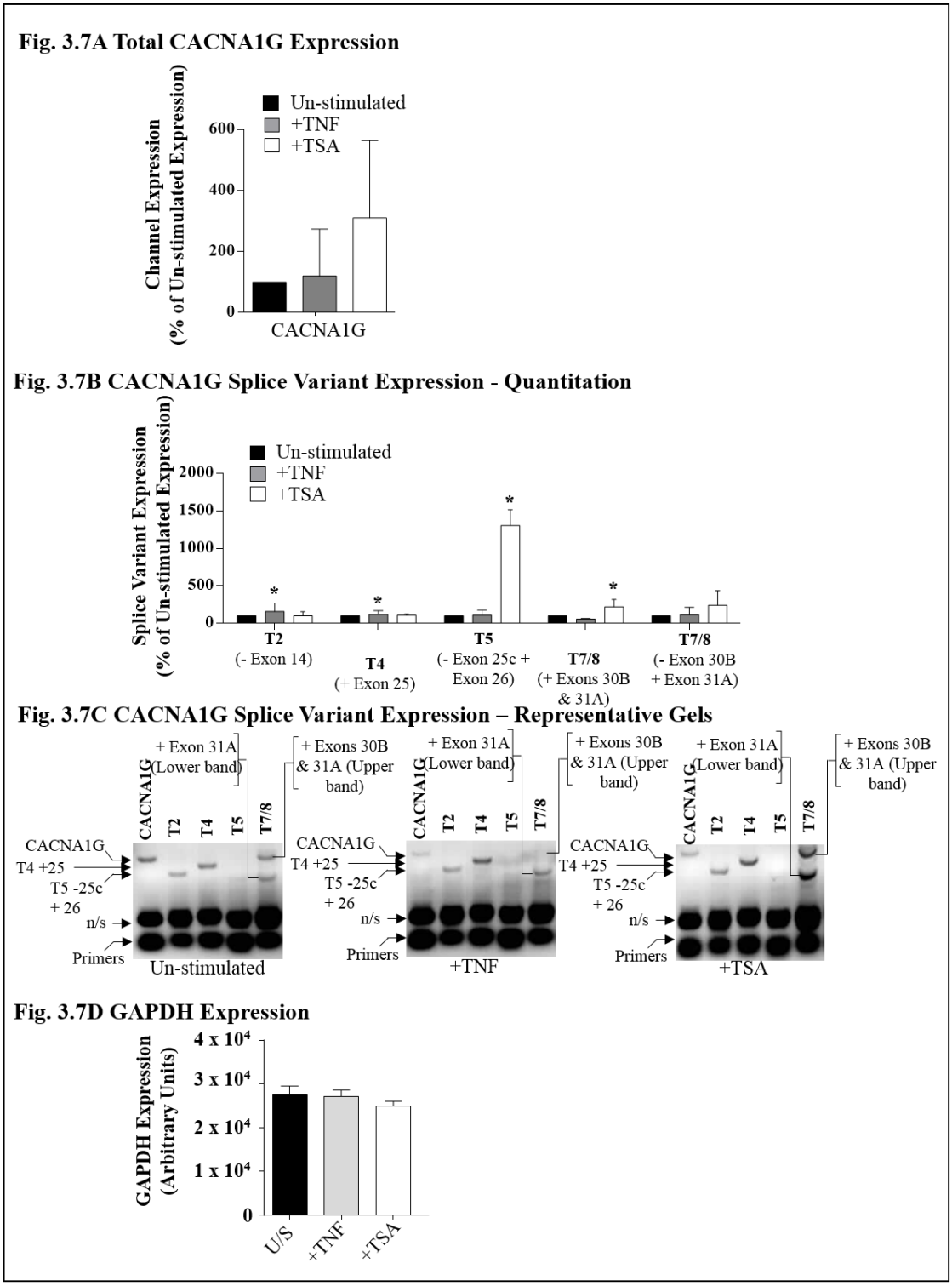


Figure 3.7 The Expression of the CACNA1G Channel and Splice Variants in Myometrial Cell Cultures

Myometrial myocyte cell cultures were stimulated with 10ng/ml TNF for 1 hour, 100ng/ml TSA for 24 hours or left un-stimulated. Total RNA was extracted, reverse transcribed and amplified using channel and splice variant specific primers. Manual quantification of the relative band intensities were used to quantify the level of expression and this was expressed as a percentage of the un-stimulated expression (n = 5). **(A)** Neither TNF nor TSA had a significant effect on the expression of the CACNA1G channel. **(B)** Myometrial cell cultures express a range of CACNA1G splice variants, TNF significantly increased the expression of T2 (-exon14) and T4 (+exon 25) while TSA significantly increased the expression of T5 (-exon25c +exon 26) and T7/8 (+exons 30B & 31A) ($p<0.05$ *). **(C)** Representative gel of the RT-PCR products. (**D)** Neither TNF nor TSA influenced the expression of GAPDH.

The exclusion of exon 25C has the effect of creating CACNA1G which are less inactivated at rest and therefore are more readily available to open in response to depolarisation of the membrane (Senatore and Spafford 2012).

TSA had little effect on the expression of any of the splice variants observed in the primary myocyte cultures. Neither treatment influenced the expression of GAPDH (Figure 3.7D).

3.3.6 The Combination of TNF or TSA Stimulus on the Expression of the CACNA1G Channel and its Splice Variants in Myometrial Cell Cultures had Differing Effects Dependant on the Splice Variant Observed.

Next, as with the KCNMA1 channel and CACNA1C it was determined if these compounds had an enhancing or diminishing effect on each other. When the cultures were exposed to TNF prior to exposure to TSA the effects observed were inconsistent. In the majority of experiments there were no bands observed on the gels which may indicate that the expression of these splice variants was significantly reduced or it may indicate a problem with the PCR reactions. Since it was not possible to confidently determine which of these explanations was correct any meaningful interpretation of the analysis was therefore impossible.

When the cultures were exposed to TSA prior to exposure to TNF the expression of the CACNA1G was increased, although this did not reach significance (Figure 3.8A, C). The expression of the splice site 4 variant containing exon 25 and the splice site 5 variant containing exon 26 and excluding exon 25C were both significantly up regulated (Figure 3.8B, C) in comparison to the un-stimulated cultures. The addition of TSA prior to TNF was observed to have an enhancing effect on both the expression of the CACNA1G and the splice site 5 variant as these were increased to a higher level than that seen after exposure to TNF alone. The addition of TSA had little additional effect on the expression of the splice site 4 variant in comparison to TNF alone. No change in GAPDH was observed with either treatment (Figure 3.8D).

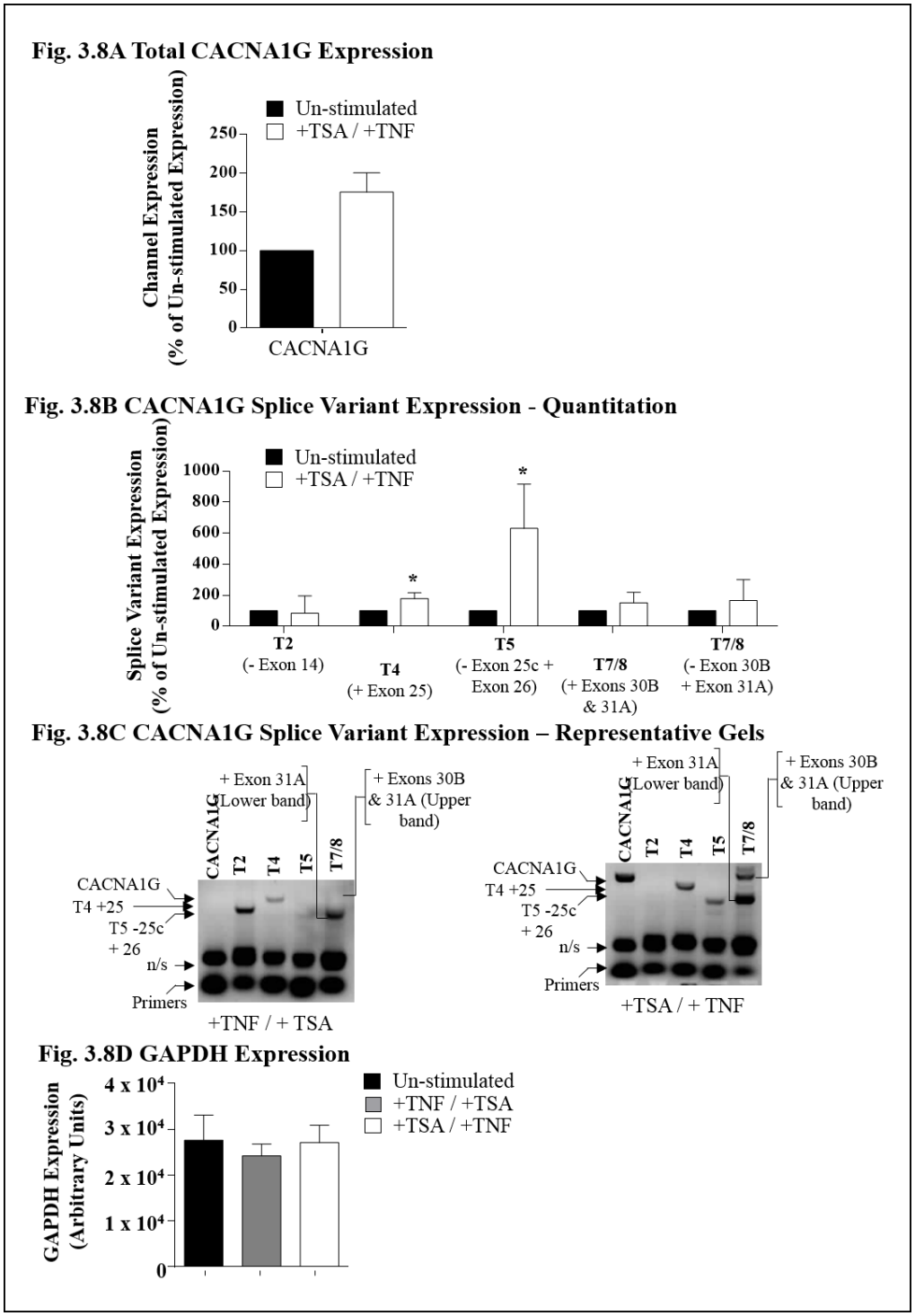


Figure 3.8 The Expression of the CACNA1G Channel and Splice Variants in Myometrial Cell Cultures

Myometrial myocyte cell cultures were stimulated with 10ng/ml TNF for 1 hour followed by 100ng/ml TSA for twenty-four hours, 100ng/ml TSA for twenty-four hours followed by 10ng/ml TNF for 1 hour or left un-stimulated. Total RNA was extracted, reverse transcribed and amplified using channel and splice variant specific primers. Further analysis was only performed on the TSA/TNF samples. Manual quantification of the relative band intensities were used to quantify the level of expression and this was expressed as a percentage of the un-stimulated expression (n = 5). **(A)** TSA/TNF had no significant effect on the expression of the CACNA1G channel. **(B)** Myometrial cell cultures express a range of CACNA1G splice variants, TSA/TNF significantly increased the expression of the T4 (+ exon 25) and T5 (-exon 25c+exon 26) splice variants ($p<0.05$ *). **(C)** Representative gel of the RT-PCR products. (**D)** Neither TNF/TSA nor TSA/TNF influenced the expression of GAPDH.

3.4 Discussion

At the end of pregnancy the myometrium switches from a state of relative quiescence, which is seen throughout pregnancy, to the contractile state characteristic of parturition. Although the molecular basis of contraction is understood, the triggers which move the myometrium from the quiescence to the contractile state seen at parturition remain to be defined.

The myometrial quiescent state is characterised by slow wave potentials where the membrane potential cycles between depolarisations and repolarisations without reaching the threshold level for action potential generation (Parkington, Tonta *et al.* 1999). However in the myometrium at parturition, these slow wave potentials become frequent and synchronised action potentials during which the membrane potential rapidly rises and falls, causing the muscle to contract (Wilde and Marshall 1988).

Both the quiescent state and the coordinated contractions are thought to be mediated through mechanisms that involve ion channels and in particular the KCNMA1 potassium, CACNA1C and CACNA1G channels.

3.4.1 Analysis of KCNMA1 Channel Expression in Myocytes

This research has demonstrated that myometrial cell cultures contain a number of different KCNMA1 channel splice variants including Mk44 and STREX-2.

Splice site 1 variant (Mk44) comprises a 132bp insertion, which has previously been described by Korovkina *et al.* Variants containing the insert (Mk44 +132bp) have diminished calcium and voltage sensitivity, the inserted sequence itself has been shown to contain both a phosphorylation and a myristylation site. This variant can also undergo proteolytic cleavage after which the N-terminal is located on the membrane while the C-terminal is held with the endoplasmic reticulum. The C-terminal is released after release of calcium from sarcoplasmic reticulum based stores and re-constitutes with the N-terminus on the membrane (Korovkina, Fergus *et al.* 2001). The presence of this variant shows that there are KCNMA1 channels with diminished calcium and voltage sensitivity within the myometrium.

Splice site 2 comprises the mutually exclusive use of exons 10 and 11 or 11 and 12, within the myocytes exons 11 and 12 are utilised. This variant can result in premature truncation of the protein. The loss of the C-terminal tail of the channel will result in the loss of the calcium sensitivity of the channel.

Splice site 3 comprises the insertion of 40aa (the utilisation of the entire of exon 19), the insertion of 4aa (the 3' truncation of exon 19) or no amino acids inserted (the skipping of exon 19). Within the myocytes the entire of exon 19 is utilised, there is no information as to the effect of the inclusion of the whole exon on channel gating kinetics.

Splice site 4 consists of the differential utilisation of exons 22 and 23. The variant present in the myocytes contained both exons 22 and 23 and so is the STREX-2 variant. The STREX-2 variant has increased mechano-sensitivity and hypoxia inhibition. Interestingly the STREX-2 insert also contains a cAMP-dependant PKA consensus motif which when phosphorylated facilitates membrane depolarisation and hence promotes the contractile phenotype of the cells.

Splice site 5 comprises the insertion or deletion of exon 29, the variant expressed within the myocytes contains exon 29. This insertion results in increased activation rates and their co-operativity with calcium is modified. It is thought these effects are due to the proximity of the splice site to the calcium bowl.

3.4.2 Analysis of CACNA1C Channel Expression in Myocytes

This research has also demonstrated that the myometrial cell cultures used as a model express pro-contractile splice variants of the CACNA1C including splice site 4 containing exon 8*, splice site 10 containing exons 30,31,32,33 and 34 and splice site 11 containing exons 40, 41, 42 and 43.

Splice site 4 is comprised of the mutually exclusive use of exons 8 and 8*. The PCR results show that it is exon 8* that is expressed in the myocytes. The expression of 8* results in a channel with decreased DHP sensitivity, rapid activation and slow deactivation kinetics (Goodwin, Leeds *et al.* 1999). The kinetics of the channel would promote contraction.

The CACNA1C splice site 10 is a complex site which involves the differential usage of exons 30, 31, 32, 33 and 34 which result in different lengths of linking loop between IV S2 and S4. The myocytes express two forms of this variant, a longer one containing exons 30, 31, 32, 33 and 34 and a shorter one containing exons 30, 31, 33 and 34. The longer form containing exon 32 correlates well with SVI3B, which has been shown to have increased excitation-contraction coupling (Huang, Xu *et al.* 2006) and so the less abundant expression of this form would decrease the excitability of the cell and promote relaxation.

Splice site 11 encompasses exons 40, 41, 42 and 43, variants in this region include use of exon 40A (exon40 – 19bp), +125bp (exon 40B), exon 41A and exon 43 +132bp or the use of exons 40, 41, 42 and 43 (Soldatov 1994). The additional 19bp found in exon 40 in comparison to exon 40A are thought to modulate the tethering of calmodulin to the C-terminal and impact on the calcium dependant inactivation of the channel (Gerhardstein, Gao *et al.* 2000). The splice site 11 variant found to be expressed in the myocytes contained exons 40, 41, 42 and 43. This variant is the same as the $\alpha 1C_{77}$ variant described by Kepplinger *et al.* (Kepplinger, Kahr *et al.* 2000). Calcium-dependant inactivation was found to be highest in the $\alpha 1C_{77}$ variant. This variant was also found to be more efficiently targeted to the cell membrane and had higher conductance and open probability than the other variants at this site (Kepplinger, Kahr *et al.* 2000). The expression of this variant would push the cells towards a more contractile state.

3.4.3 Analysis of CACNA1G Channel Expression in Myocytes

Finally, the results demonstrated that myometrial cell cultures used as a model express a number of different CACNA1G splice variants including omission of exon 14, utilisation of exon 25 with no additions and utilisation of exon 26. These variants are again predominantly pro-contractile.

The CACNA1G splice site 2 involves the insertion or deletion of exon 14, only the deleted exon 14 variant was found to be present in the myocytes. Expression of this variant will result in the loss of a PKC phosphorylation site. PKC has been demonstrated to stimulate CACNA1G activity and so the loss of a PKC site would reduce the stimulatory effect of

PKC on the channel (Park, Kang *et al.* 2006). The inclusion of exon 14 with either exon 25C or exon 26 results in channels with increased inactivation rates. As exon 14 is not expressed in these cultures these channel will not have this increased inactivation rates and so will support contraction.

The CACNA1G splice site 4 involves the possible use of a number of splice acceptor sites on exon 25. Exon 25 contains 3 splice acceptor sites termed 25A, 25A' and 25A'' however none of these have been utilised in the myocyte CACNA1G. Use of these splice acceptor sites can result in non-ion conducting channels, so the lack of use of these sites is a good indicator that the CACNA1G channels are able to conduct calcium ions through their pore. Variants containing exon 25 have been shown to activate and inactivate at more hyperpolarised potentials (Hawkins and Kearney 2012). This will have the effect of allowing these channel to open at more negative membrane potentials and so will support contraction.

The CACNA1G splice site 5 comprises the mutually exclusive use of exon 25C or 26. Within the myocytes it was exon 26 which was found to be expressed. Channels expressing exon 26 have been shown to activate at midpoint potentials and a mid-range steady state midpoint (Chemin, Monteil *et al.* 2001, Jagannathan, Punt *et al.* 2002, Bertolesi, Walia Da Silva *et al.* 2006, Emerick, Stein *et al.* 2006) The exclusion of exon 25C has the effect of creating CACNA1G which are less inactivated at rest and therefore are more readily available to open in response to depolarisation of the membrane (Senatore and Spafford 2012).

The CACNA1G splice site 7/8 involves the use of a splice donor site in exon 30 (30B) and / or the use of a Splice acceptor site in 31 (31A). Within the myocytes there were splice variants expressing both exons 30B and 31A and variants expressing just exon 31A. Inclusion of exon 30B results in channels with modified voltage dependant activation, and inclusion of exon 31A results in prematurely truncated protein.

3.4.4 The Effect of TNF

The initiation of the contractile state seen at parturition is characterised by an increase in pro-inflammatory cytokines such as IL-1β, IL-6 and TNF (Romero, Mazor *et al.* 1992,

Opsjln, Wathen *et al.* 1993, Keelan, Marvin *et al.* 1999, Osman, Young *et al.* 2003). The data from the collagen gel contraction studies demonstrating the contractile action of TNF and the pro-relaxant function of TSA are in broad agreement with the published data on the effects of TNF and TSA on contractility of isolated smooth muscle strips (Sadowsky, Adams *et al.* 2006, Fitzgibbon, Morrison *et al.* 2009, Europe-Finner, Taggart *et al.* 2013, Webster, Waite *et al.* 2013).

In terms of calcium flux, using hippocampal neurons, TNF stimulation has been shown to increase calcium current density by around 20% and this increase can be attributed to an increase in CACNA1C current (Furukawa and Mattson 1998). Furukawa *et al.* went on to show that this increase was dependant on NFκB activation as inhibition of NFκB resulted in repression of these calcium current increases.

Calcium sensitization has also been suggested as playing a role in the switch from quiescence to contractility. Calcium sensitization is the phenomenon by which a given concentration of intracellular calcium results in a larger than expected force of contraction (Ratz, Berg *et al.* 2005, Arthur, Taggart *et al.* 2007, Wray 2007). TNF has been shown to enhance Ca^{2+} responsiveness ten fold in airway smooth muscle. It has been shown that TNF may cause this increased Ca^{2+} sensitivity through inducing increased IP3 turnover and hence increased release of Ca^{2+} from intracellular stores. Another putative mechanism for calcium sensitization is via the loss or reduction in calcium sensitivity of calcium activated potassium channels. A reduction in, for example, KCNMA1 channel calcium sensitivity would result in a reduction in the repolarising current supplied by these channels and hence an increase in contractility.

This data demonstrates that TNF induced a small but statistically significantly increase in the transcription of the KCNMA1 channel. This initially appears to be at odds with TNF promoting contraction, however, an up regulation in transcription does not always correspond to an up regulation of translation. Kim *et al.* demonstrated this lag between KCNMA1 RNA and protein up-regulation in chick cochlear development and determined that it was due to delays in protein synthesis and trafficking / scaffolding of the channel subunits (Kim, Beyer *et al.* 2010). A similar delay may be occurring in the myometrium.

The expression of the KCNMA1 STREX-2 variant was unchanged with TNF stimulation, however, $G_{\alpha s}$ is known to decrease during parturition (Europe-Finner, Phaneuf *et al.* 1994) and as a result of this the level of PKA would decrease. This decrease in PKA would result in decreased phosphorylation of the KCNMA1 channel, but any phosphorylation in the presence of STREX-2 would facilitate membrane depolarisation and support contraction (Tian, Coghill *et al.* 2004). This support of contraction would decrease as the levels of PKA decrease. Hypothetically this could lead to support for contraction while PKA levels are high at the start of parturition, then a gradual switch to support quiescence as the PKA levels drop leading to termination of parturition.

TNF also resulted in a significant up regulation of the CACNA1C while the overall splice variant profile was unchanged.

TNF did not affect overall expression of the CACNA1G. It did, however, induce a small but statistically significantly increase in the expression of the splice site 2 variant without exon 14. The levels of PKC have been shown to increase towards the onset of parturition (Li, Zhang *et al.* 2012) and PKC phosphorylation has been shown to activate the CACNA1G (Park, Kang *et al.* 2006). Therefore, the loss of a PKC phosphorylation site may act to limit the activation of the CACNA1G at this juncture.

TNF also caused an increase in the expression of the splice site 4 variant containg exon 25. This variant results in channels which open more easily at more negative membrane potentials and as such these variants may be key to the myometriums ability to generate spontaneous phasic contractions. The generation of these spontaneous phasic contractions is due to the ability of the cell to fire regenerative action potentials (Lee, Ahn *et al.* 2009). Regenerative action potentials are created when a channel opening at a low threshold is able to initiate the opening of enough channels to initiate an action potential. CACNA1Gs with the ability to open at more negative membrane potentials due to the presence of these variants will allow calcium to enter the cell and so will push the membrane potential to more positive potentials which will then allow the CACNA1Cs to open and generate an action potential.

3.4.5 The Effect of TSA

The transition of the myometrium from the quiescent state to the contractile state seen at parturition has been shown to be facilitated by the down regulation of the hCG/LH receptors. Phillips *et al.* demonstrated that TSA promoted the transcriptional activation of the hCG/LH receptor gene and hence the maintenance of quiescence (Phillips, Tyson-Capper Née Pollard *et al.* 2005, Moynihan, Hehir *et al.* 2008). It has also been shown that the maintenance of myometrial quiescence is facilitated by the expression of $G_{\alpha s}$. Acetylation of the $G_{\alpha s}$ promoter by CBP is necessary for its expression within the myometrium and therefore TSA may also act by preventing the deacetylation of the $G_{\alpha s}$ promoter and hence promote the expression of $G_{\alpha s}$ and the maintenance of myometrial quiescence (Webster, Waite *et al.* 2013).

This research suggests a further possible mechanism for the pro-quiescent action of TSA via its effect on the expression of the KCNMA1 potassium and CACNA1C channels. After TSA stimulation the CACNA1C expressed variant containing exon 8 and exon 8*, the presence of variants containing exon 8 would result in a less contractile phenotype. TSA resulted in a significant down regulation of both channels. This reduction in channel transcription would result in cells much less responsive to calcium and hence less contractile.

TSA did not affect the overall expression of the CACNA1G, however the expression of splice variants containing exon 26 and those containing both exons 30B and 31A were significantly up-regulated. The IIIS6 portion of the CACNA1G calcium channel has a role in its inactivation, exon 26 lies within this region and therefore may affect the inactivation of the channel. The C-terminal tail region of the CACNA1G channel has been shown to be responsible for the fast inactivation of the channel (Staes, Talavera *et al.* 2001), therefore, inclusion of exon 31A which can result in premature truncation of the protein may alter the inactivation of the channel. Although further research is need to fully explore the effects of these splice variants on myometrial contractility, the documented effects of these splice variants would suggest that they would support a more relaxed phenotype.

3.4.6 The Combined Effect of TNF and TSA

If histone deacetylase inhibitors are to be developed as potential tocolytics it is important to understand their effect on the KCNMA1 channel, CACNA1C and CACNA1G when it is introduced either before and after the cytokine influx characteristic of parturition.

When the primary myocytes were stimulated with TSA following TNF stimulation there was a significant down-regulation of both the KCNMA1 and CACNA1C channels which was greater than that seen with TSA alone. When the myocytes were stimulated with TNF following TSA stimulation TNF seems to be able to partially recover the expression of the KCNMA1 channel in comparison to that seen with TSA alone. With the CACNA1C when added second TNF is only able to prevent the further down-regulation which is seen when the stimulants are added in the reverse order.

The expression of the CACNA1C variant containing exon 8, seems to be dependent on TSA stimulation. This variant is expressed whether TSA used before or after TNF stimulation indicating that acetylation may have an important role in the expression of this variant. HDAC inhibition has been shown to alter splicing of ~700 genes and to reduce the interaction between splicing regulator SRp40 and the target fibronectin exon (Hnilicova 2010). A similar number of genes were observed to change in the myometrium upon TSA administration as shown by Karolczak-Bayatti *et al.* (Karolczak-Bayatti, Sweeney *et al.* 2011). As TSA is a general HDAC inhibitor this may explain the switch in splice variant expression.

The effect on the expression of both the long and short variants at splice site L10 of the CACNA1C seem to be dependent on the order of the two stimulations. When TNF stimulation occurs first the expression of these two variants is similar to that seen in TNF stimulation alone. However, when TSA stimulation occurs first the expression of these two variants is similar to that seen with TSA alone. The converse is the case for the KCNMA1 splice variant containing exon 19 (M3) When TNF stimulation follows TSA stimulation, it appears that TNF blocks the TSA induced down regulation of this variant, however when TSA stimulation follows TNF stimulation, TNF is not able to completely block the down-regulation of this variant although it is less down-regulated than is seen with TSA alone.

There are also splice variants were the combined stimulation results in a greater effect than either stimulation on its own. This is the case for the KCNMA1 STREX-2 variant and the KCNMA1 variant containing exon 29, where TNF stimulation followed by TSA stimulation results in a significant up-regulation of both variants.

Webster *et al.* demonstrated that TNF could overcome TSA induced myometrial relaxation in smooth muscle strips (Webster, Waite *et al.* 2013). When combined with the data included here this would indicate that the effect of TNF on the KCNMA1 channel, the KCNMA1 Splice site M1 +132bp, M3 exon 19, the CACNA1C splice site L10/11, the CACNA1G splice sites T4 and T6 variants are key to inducing contraction. It would be necessary, however, to carry out patch clamp recordings and/or collagen gel contractions assays to determine the overall electrical/ contractile effect of these combined stimuli on the cells to verify this.

3.5 Conclusion

This research suggests that increasing TNF levels at parturition could promote the increased calcium sensitivity of the myometrium through the expression of KCNMA1 channel slice variants documented to have decreased calcium and voltage sensitivity alongside CACNA1C calcium channels documented to have rapid activation, slow deactivation kinetics and increased excitation-contraction coupling.

The combined effect of TNF on the increase in transcription of CACNA1C channels and the expression of pro-contractile KCNMA1 channel variants may serve to tip the fine balance of the channels allowing action potentials to be generated and propagated across the tissue resulting in contraction.

TSA was seen to promote a relaxatory effect on the cells, this is appears to be through the significant reduction in transcription of both channels resulting in cells that are less excitatory.

As these channels will only affect the contractility of the myocytes once they are localised to the cell membrane the next step in this research would be to examine the expression and localisation of the channel proteins and the effect of exposure to TNF and TSA on

this expression and localisation. A further area worthy of investigation is whether the expression of the different splice variants observed in the RNA data resulted in the expression of different protein isoforms. These two areas will be the focus of the following chapter.

Chapter 4: Localisation of the KCNMA1, CACNA1C and CACNA1G Proteins in Myometrial Cell Cultures Results and Discussion

4.1 Introduction

In Chapter 3, the collagen gel contraction assay demonstrated that TNF caused significant contraction in myometrial cell cultures and TSA caused relaxation in these cultures. Next, the expression of the KCNMA1, CACNA1C and CACNA1G channels were examined to determine if the contractile effect of TNF or the relaxatory effect of TSA was mediated via changes in the amount of channels expressed or in the splice variant profile of the expressed channels. The results of these studies indicated that non-labouring term pregnant myocytes expressed a number of different splice variants. These included the KCNMA1 Mk44+132bp, Mk44-132bp, M2 (exon 11+12), M3 (exon 19), M4 (STREX2) and M5 (exon 29) variants, the CACNA1C L4 (exon 8* or Exon 8), L10 (exons 30, 31, 33 and 34 or exons 30, 31, 32 and 34) and L11 (40, 41, 42 and 43) variants and the CACNA1G T2 (exon 14 deleted), T4 (exon 25), T6 (exon 26) and T7/8 (exon 31A or exon 30B and 31A) variants. Upon TNF stimulation both the KCNMA1 and CACNA1C expression was significantly up-regulated and upon TSA stimulation both channels were significantly down regulated and the splice variant profile of the CACNA1C and CACNA1G was significantly altered.

The KCNMA1, CACNA1C and CACNA1G are membrane-spanning proteins which form pores that pass from one side of the membrane to the other. These pores open and close in response to particular signals, when open these pores allow the passage of ions either into or out of the cell. Each ion channel has specific gating characteristics meaning only the correct ion can pass through the appropriate pore. The charge of the ion passing through the channel and the direction in which the ion is moving (either into or out of the cell) will affect the potential of the cell membrane, this then triggers further voltage sensitive channels to open or close. Within smooth muscle cells this propagated signal will then lead to either contraction or relaxation of the cell.

In very simple terms an increased number of KCNMA1 channels would mean an increased number of potassium ions leaving the cell for any given activation and this will have the effect of reducing the membrane potential and returning the cell to the resting state more quickly. In the same way an increased number of CACNA1C and/or CACNA1G would mean an increased number of calcium ions entering the cell for any given activation and this will have the effect of increasing the membrane potential and

generating an action potential more quickly. The caveat to this would be that the channels are housed within the plasma membrane and have the same gating kinetics, however, this is unlikely to be the case *in vivo*.

The KCNMA1 channel has been shown to undergo splicing and expression of these different splice variants can have diverse effects on the channel from altering the gating of the channel, the introduction of cleavage sites and even truncation of the channel protein. One such splice variant, Mk44, which was expressed in the myometrial cell cultures and has been described by Korovkina *et al.* undergoes proteolytic digest and after this digest the N-terminus of the protein is expressed on the cell membrane while the C-terminus is intracellular and localised specifically with the endoplasmic reticulum (Korovkina, Brainard *et al.* 2006). After calcium release from the sarcoplasmic reticulum the C-terminus translocates to the cell membrane and non-covalently associates with the N-terminus. When the C-terminus is associated with the N-terminus the channel has decreased calcium and voltage sensitivity demonstrating that the C-terminus has an inhibitory effect on channel gating. Therefore, it is important to determine the location of both termini of the KCNMA1 channel in order to understand the potential effect the channel will have on membrane potential.

The C-terminal KCNMA1 antibody utilised had been used for western blotting previously by a number of research groups (Alioua, Lu *et al.* 2008, Shi, Liu *et al.* 2013) and had been shown to be specific. The N-terminal KCNMA1 antibody had been tested in western blot against the recombinant peptide used as an immunogen but there was no data on its ability to detect endogenous protein, therefore less confidence can placed in the data obtained from the use of this antibody.

The CACNA1C channel has been shown to exist in two forms, a 220kDa protein and a 190kDa protein. The shorter channel is formed as a result of the cleavage of the C-terminal region. Calpain has been shown to be responsible for this cleavage (Hell, Westenbroek *et al.* 1996) which occurs in exon 42 between splice variants 11 and 12. Interestingly, it has been shown that calmodulin binds calpain substrates and in doing so can regulate calpain-mediated proteolysis causing inhibition of this process (Wang, Villalobo *et al.* 1989, Iwamoto, Lu *et al.* 2010). An insertion at splice site 11 has been shown to modulate calmodulin binding and therefore this splice site could have an effect

on the cleavage of the channel. Once cleaved, the C-terminus can either co-localise with the N-terminus of the channel or it can translocate to the nucleus. The loss of the C-terminus of the channel results in a 4-6 fold increase in ion conductance in comparison to the full length channel. So similar to the KCNMA1 channel the C-terminus of the CACNA1C channel has an inhibitory effect. The primary myocytes were shown to express a splice site 11 variant containing exons 40, 41, 42 and 43, this variant may have an effect on the cleavage of the CACNA1C. The proteolytic processing of the C-terminus of the CACNA1C has been shown to generate fragments of 56, 48 and 30kDa (Figure 4.1; Gao, Cuadra *et al.* 2001), which, when they associate with the main body of the channel protein and have a role in inhibiting channel function.

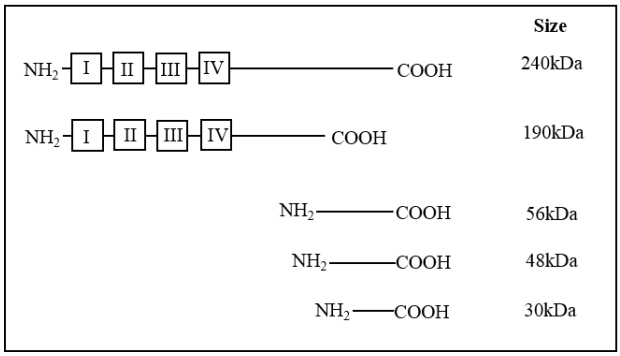


Figure 4.1 Diagrammatic Representation of the Generation of the Various CACNA1C C-terminal Fragments

Therefore, it is again important to determine the location of both termini of the CACNA1C channel in order to understand the potential effect the channel will have on membrane potential.

The N-terminal CACNA1C antibody utilised had been used for western blotting previously by a number of research groups (Li, Zhang *et al.* 2013, O'Connell, Musa *et al.* 2015) and had been shown to be specific. The C-terminal CACNA1C antibody had not been tested in western blot, therefore less confidence can placed in the data obtained from the use of this antibody.

Immunocytochemistry staining of the myometrial cells, in Chapter 3, showed evidence of nuclear localisation for both the KCNMA1 and CACNA1C channels. Although nuclear localisation has been previously described for the CACNA1C channel (Gomez-Ospina, Tsuruta *et al.* 2006, Gomez-Ospina, Panagiotakos *et al.* 2013), nuclear localisation of the

KCNMA1 channel was a novel finding. It is important to determine if this nuclear localisation was an artefact from the staining protocol or if the KCNMA1 channel does indeed localise to the nucleus. If the KCNMA1 channel is shown to localise to the nucleus by western blot analysis it is also important to determine which portion of the channel (N- or C-terminus or both) is found within the nucleus. For the above reasons both an antibody specific to the N-terminus and one specific to the C-Terminus were used for western blotting for both the KCNMA1 and the CACNA1C channels.

Studies of the CACNA1G protein within human male germ cells and human cerebellum cDNA has revealed that the CACNA1G protein exists in a number of isoforms, including a longer 262kDa protein and a shorter 249kDa protein (Monteil, Chemin *et al.* 2000, Jagannathan, Punt *et al.* 2002). These isoforms have been shown to be the result of alternative splicing, with the 249kDa form resulting from the use of an alternative 5'-splice donor site on exon 25 combined with the acceptor site on exon 27 (Figure 4.2A; Monteil, Chemin *et al.* 2000). Alternatively, the 262kDa protein results from the use of a 5'-splice donor site on exon 25 combined with the acceptor site on exon 26 (Figure 4.2B; Monteil, Chemin *et al.* 2000). The 249kDa form has the most positive voltage range for steady state inactivation (Monteil, Chemin *et al.* 2000) meaning that the CACNA1G inactivates more slowly allowing more calcium to enter the cell. Within the primary myocyte cultures the cells were observed to express splice variants containing exon 25 and exon 26 and so correlate well with the 262kDa form of the protein, however, it is still pertinent to identify which isoforms of the CACNA1G are expressed by the primary myocytes.

The C-terminal CACNA1G antibody utilised had been used for western blotting previously and had been shown to be specific (Nguyen, Biet *et al.* 2013).

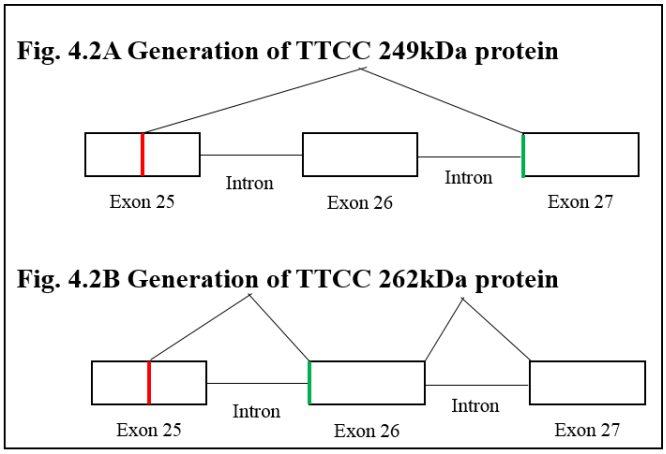


Figure 4.2 Diagrammatic Representation of the Generation of the 262kDa and 249kDa CACNA1G Isoforms

The aim of this chapter was, therefore, to measure the expression of the KCNMA1 channel, CACNA1C and CACNA1G proteins and to identify any alternative isoforms expressed within the primary myocytes. Further to this, the localisation of the KCNMA1, CACNA1C and CACNA1G proteins and their isoforms within the cell was then examined. Finally, the effect of TNF and TSA on the expression and localisation of the KCNMA1 channel, CACNA1C and CACNA1G was studied.

4.2.2 Gβ Loading Control.

In order to be certain that any changes in band intensity visible on the western blots were as a result of the different stimulations and not as a result of errors, either when quantifying the protein or when loading the gel, a western blot was ran utilising a housekeeping protein which is unaffected by the different stimulations.

The housekeeping protein chosen was Gβ. This is routinely used in the research group as a control and is not part of either the myometrial relaxation or contraction pathways (Phaneuf, Europe-Finner *et al.* 1993, Europe-Finner, Phaneuf *et al.* 1994, Salomonis, Cotte *et al.* 2005). All channel western blots were carried out with the same protein samples with the Gβ control serving experiments for KCNMA1, CACNA1C and CACNA1G channels. No change in Gβ was observed with any treatment in any of the protein fractions (Figure 4.3)

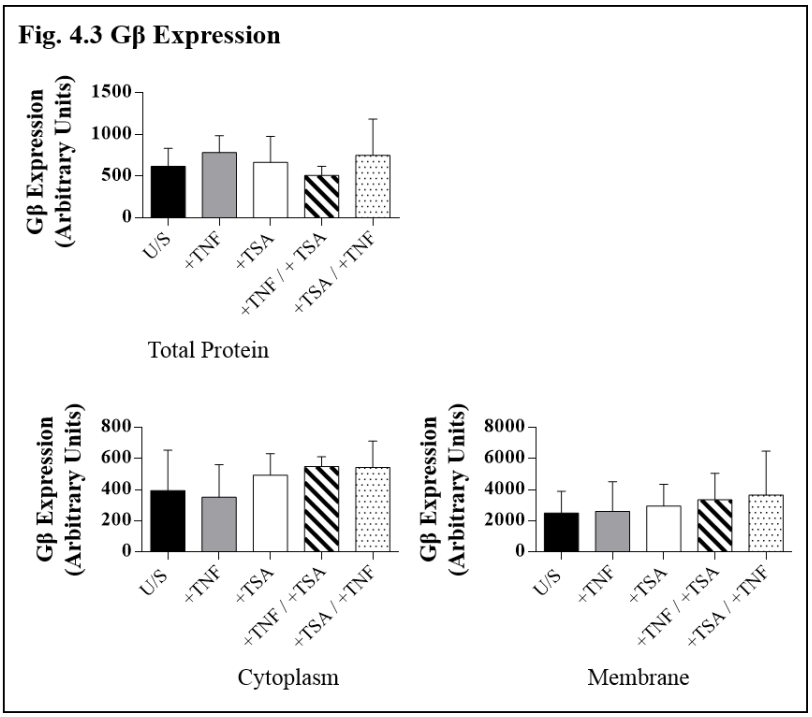


Figure 4.3 Gβ Expression
Myometrial cell cultures were stimulated with 10ng/ml TNF, 100ng/ml TSA, 10ng/ml TNF followed by 100ng/ml TSA (+TNF/+TSA), 100ng/ml TSA followed by 10ng/ml TNF (+TSA/+TNF) or left un-stimulated. Protein was extracted, separated by SDS PAGE and western blotted. Manual quantification of the relative band intensities were used to quantify the level of expression and this was expressed as arbitrary units. None of the treatments had any effect on Gβ expression in any of the fractions.

4.3 The KCNMA1 Channel

4.3.1 N-Terminal KCNMA1 Protein and Protein Fragments are Expressed in Myometrial Cell Cultures and are Unaffected by Either TNF or TSA

This present research sought to determine if TNF or TSA could influence expression of the KCNMA1 protein. Total cellular protein was isolated, separated by SDS PAGE and subsequently western blotted with an antibody directed to the N-terminus of the KCNMA1 protein.

Blotting with the N-terminal antibody did not detect full length KCNMA1 protein as evidenced by the lack of band at 138kDa (Figure 4.4A) this may indicated that the protein is N-terminally truncated. The N-terminal antibody did detect two fragments of the KCNMA1 protein one at 65kDa and one at 50kDa (Figure 4.4A). This would indicated that there are KCNMA1 proteins which have had the C-terminal region cleaved from the body of the channel expressed within the primary myocyte cultures. Cleavage of the C-

terminal region of the KCNMA1 protein has been shown to increase the channels calcium and voltage sensitivity and thus would support relaxation. The 65kDa band corresponds to a band reported by Knaus *et al.* They determined that this band was produced when the KCNMA1 channel was cleaved between residues 640 and 690 (Knaus, Eberhart *et al.* 1995).

When cultures were subsequently exposed to either TNF or TSA, neither had any significant effect on either the KCNMA1 protein level or the fragments expressed in comparison to un-stimulated samples (Figure 4.4A).

4.3.2 Membrane Localisation of the 50kDa N-Terminal KCNMA1 Protein Fragment Expressed in Myometrial Cell Cultures is Significantly Increased by TNF

Next it was determined if these compounds had an effect on the localisation of the KCNMA1 protein or protein fragments. Protein was isolated by sub cellular fractionation, separated by SDS PAGE and subsequently western blotted with an antibody directed to the N-terminus of the KCNMA1 protein.

Blotting with the N-terminal antibody revealed the same complement of fragments as was detected within the whole cell protein preparations (65kDa and 50kDa; Figure 4.4B, C). Full length (138kDa) KCNMA1 channel was also detected in the cytoplasmic fraction (Figure 4.4B).

When cultures were subsequently exposed to either TNF or TSA, TNF resulted in a significant increase in the membrane bound 50kDa KCNMA1 in comparison to unstimulated samples (Figure 4.4C; $p<0.05$). The increase in this fragment would appear to be at odds with action of TNF as this fragment would support relaxation due to the lack of the inhibitory C-terminal tail region. TNF did not have any significant effect on the localisation of the other fragments and TSA had no significant effect on any of the fragments observed in comparison to the un-stimulated samples.

Fig. 4.4 KCNMA1 N-terminal Domain

Fig. 4.4A Total Cellular KCNMA1 Protein – Representative Gel and Quantitation

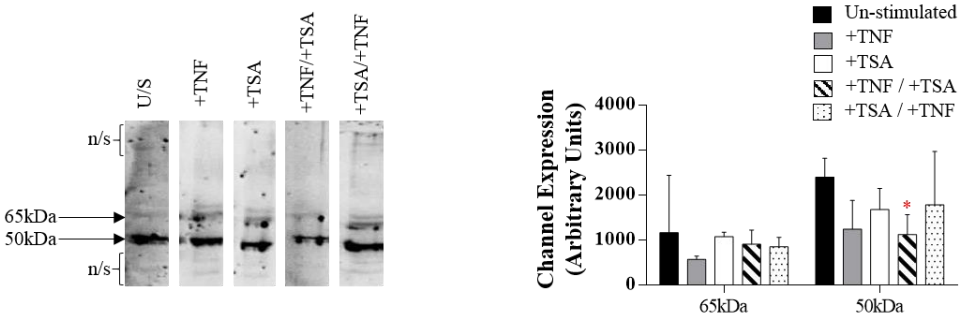


Fig. 4.4B KCNMA1 Cytoplasmic Localisation – Representative Gels and Quantitation

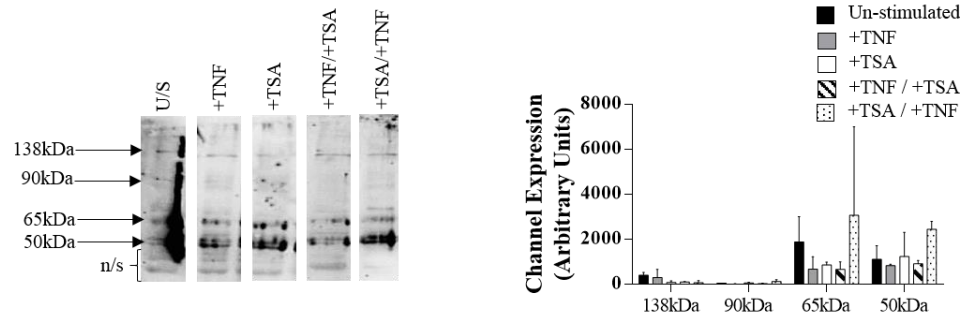


Fig. 4.4C KCNMA1 Membrane Localisation – Representative Gels and Quantitation

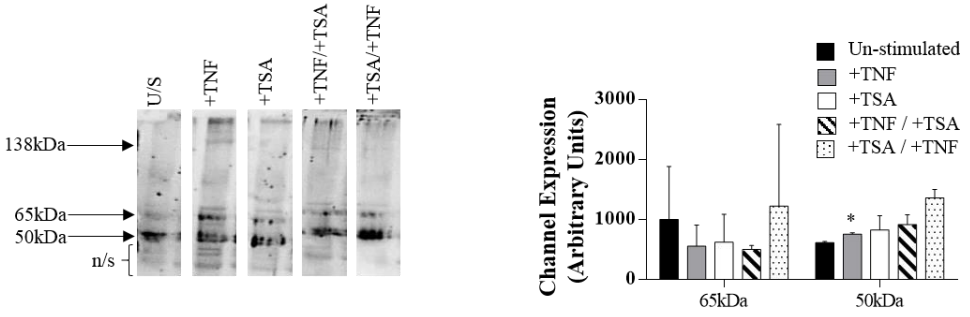


Figure 4.4 The Expression of the KCNMA1 Channel Protein in Myometrial Cell Cultures Utilising an N-terminal Antibody

Myometrial myocyte cell cultures were stimulated with 10ng/ml TNF for twenty-four hours, 100ng/ml TSA for twenty-four hours, 10ng/ml TNF for 1 hour followed by 100ng/ml TSA for twenty-four hours (+TNF/+TSA), 100ng/ml TSA for twenty-four hours followed by 10ng/ml TNF for 1 hour (+TSA/+TNF) or left un-stimulated. Protein was extracted, separated by SDS PAGE and western blotted. Manual quantification of the relative band intensities were used to quantify the level of expression and this was expressed as arbitrary units (n = 3). **(A)** Within total cellular protein myometrial cell cultures express two KCNMA1 proteins (right panel), +TNF/+TSA caused a significant down regulation of the 50kDa fragment in comparison to TSA ($p<0.05$*). **(B)** Cytoplasmic localisation of the KCNMA1 protein (right panel), no treatment had any effect KCNMA1 protein expression (left panel). **(C)** Membrane localisation of the KCNMA1 protein (right panel), TNF cause a significant increase in membrane localisation of the 50kDa fragment in comparison to un-stimulated ($p<0.05$*), none of the other treatments had any effect KCNMA1 protein expression (left panel).

4.3.3 Localisation of the KCNMA1 Protein and Protein Fragments Expressed in Myometrial Cell Cultures is Unaffected by Combined TSA and TNF Stimulation.

Next it was determined if these compounds had an enhancing or diminishing effect on each other.

When the cultures were exposed to TNF prior to exposure to TSA and then blotted with the N-terminal antibody there was no significant effect on the expression of the KCNMA1 channel protein, protein fragments or their subcellular localisation in comparison to un-stimulated samples (Figure 4.4A, B, C). However, there was a significant decrease in the expression of the 50kDa fragment within the total cellular protein in comparison to exposure to TSA alone (Figure 4.4A; $p<0.05$). This fragment was expressed at a level similar to the TNF samples, indicating that TSA does not affect the effect of TNF on this fragment.

When the cultures were exposed to TSA prior to TNF and blotted with N-terminal antibody there was again no significant effect on the expression of the KCNMA1 channel protein, protein fragments or their subcellular localisation in comparison to un-stimulated samples (Figure 4.4A, B, C).

4.3.4 C-Terminal KCNMA1 Protein and Protein Fragments are Expressed in Myometrial Cell Cultures and are Unaffected by Either TNF or TSA

This present research sought to determine if TNF or TSA could influence expression of the KCNMA1 protein. Total cellular protein was isolated, separated by SDS PAGE and subsequently western blotted with an antibody directed to the C-terminus of the KCNMA1 protein.

When the C-terminal antibody was utilised, a 138kDa protein was detected (Figure 4.5A), which represents full length KCNMA1 protein. This is in apparent disagreement with the results from the N-terminal antibody, however, this discrepancy could be explained by differences in the efficiency of antibody binding between the N- and C-terminal antibodies.

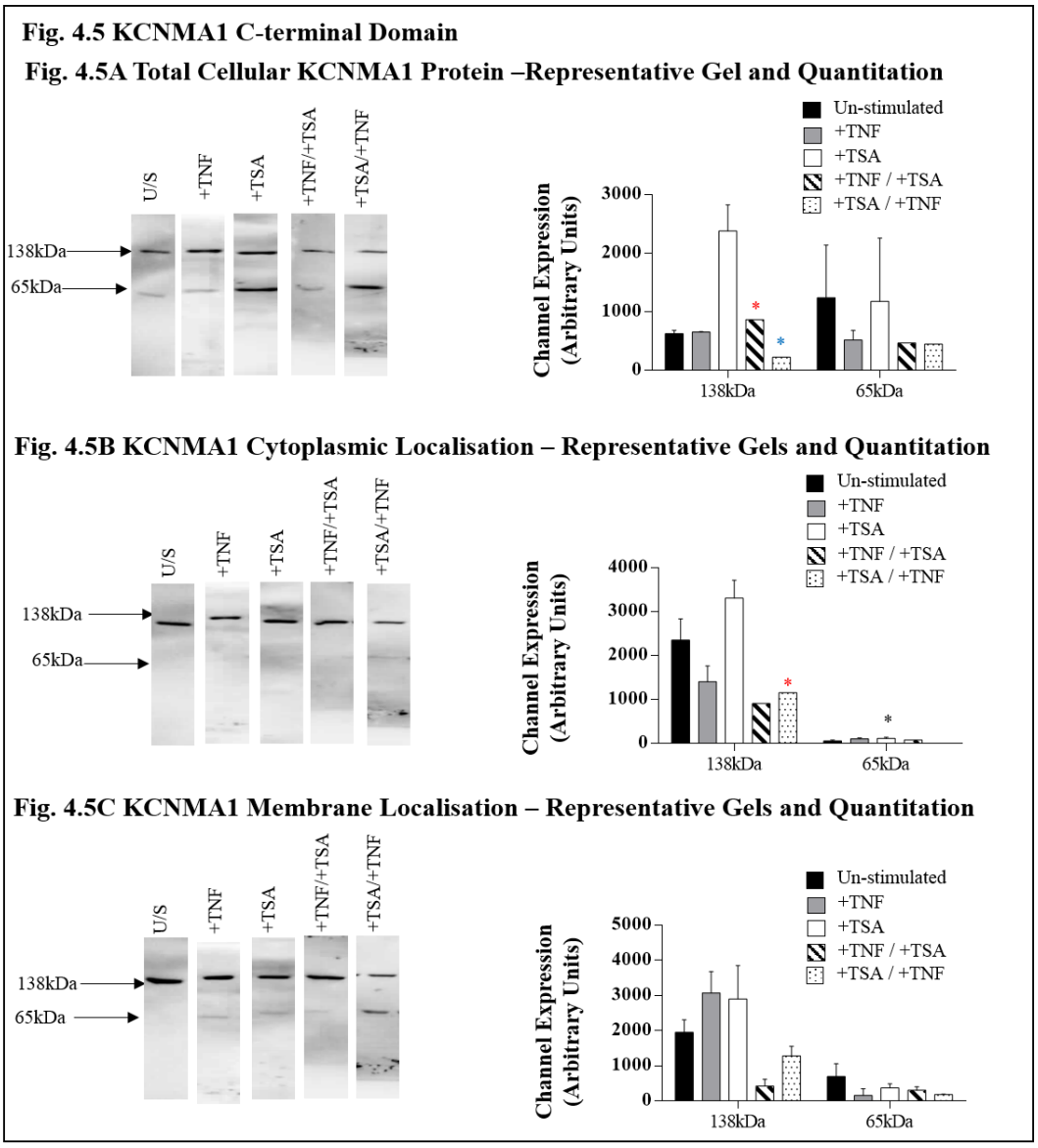


Figure 4.5 The Expression of the KCNMA1 Channel Protein in Myometrial Cell Cultures Utilising a C-terminal Antibody

Myometrial myocyte cell cultures were stimulated with 10ng/ml TNF for twenty-four hours, 100ng/ml TSA for twenty-four hours, 10ng/ml TNF for 1 hour followed by 100ng/ml TSA for twenty-four hours (+TNF/+TSA), 100ng/ml TSA for twenty-four hours followed by 10ng/ml TNF for 1 hour (+TSA/+TNF) or left un-stimulated. Protein was extracted, separated by SDS PAGE and western blotted. Manual quantification of the relative band intensities were used to quantify the level of expression and this was expressed as arbitrary units (n = 3). **(A)** Within total cellular protein myometrial cell cultures express two KCNMA1 proteins (right panel), +TNF/+TSA caused a significant down regulation of the 138kDa fragment in comparison to TSA ($p<0.05$*). +TSA/+TNF cause a significant down regulation of the 138kDa protein in comparison to un-stimulated ($p<0.05$*) and TNF ($p<0.05$*) **(B)** Cytoplasmic localisation of the KCNMA1 protein (right panel), TSA caused a significant up regulation of the 65kDa fragment in comparison to un-stimulated ($p<0.05$*) and +TSA/+TNF caused a significant down regulation of the 138kDa protein in comparison to TSA ($p<0.05$*). **(C)** Membrane localisation of the KCNMA1 protein (right panel), no treatment had any effect KCNMA1 protein expression (left panel).

A second possible explanation is that the protein detected by the C-terminal antibody is lacking a small portion of the N-terminus, this would mean that it would not be detected

by the N-terminal antibody, while at the same time the reduction in overall protein size would not be immediately apparent. The C-terminal antibody also detected a 65kDa KCNMA1 protein fragment (Figure 4.5A). This fragment is lacking the N-terminal region, utilising the binding site of the C-terminal antibody it is possible to predict that this fragment of the protein is comprised of just the C-terminal tail region and so lacks the membrane spanning domains and importantly will not contain the channel pore and so cannot conduct ions.

When cultures were subsequently exposed to either TNF or TSA, neither had any significant effect on either the KCNMA1 protein level or the fragments expressed in comparison to un-stimulated samples (Figure 4.5A).

4.3.5 Cytoplasmic Localisation of the 65kDa C-Terminal KCNMA1 Protein Fragment Expressed in Myometrial Cell Cultures is Significantly Increased by TSA

Next it was determined if these compounds had an effect on the localisation of the KCNMA1 protein or protein fragments. Protein was isolated by sub cellular fractionation, separated by SDS PAGE and subsequently western blotted with an antibody directed to the C-terminus of the KCNMA1 protein.

When the protein was probed with the C-terminal antibody both full length (138kDa) KCNMA1 protein and the 65kDa N-terminally truncated fragment were detected within the cytoplasmic and membrane bound fractions (Figure 4.5B, C).

When cultures were subsequently exposed to either TNF or TSA, TNF did not have any significant effect on the localisation of any of the fragments. TSA, resulted in a small but statistically significant increase in the cytoplasmic localisation of the 65kDa fragment but had no significant effect on any of the other fragments observed in comparison to the un-stimulated samples.

4.3.6 KCNMA1 138kDa Protein Expressed in Myometrial Cell Cultures is Significantly Decreased by Stimulation with TSA Followed by TNF.

Next it was determined if these compounds had an enhancing or diminishing effect on each other.

When the cultures were exposed to TNF prior to exposure to TSA and then blotted with the C-terminal antibody there was no significant effect on the expression of the KCNMA1 channel protein, protein fragments or their subcellular localisation in comparison to un-stimulated samples (Figure 4.5A, B, C). However, there was a significant decrease in the expression of the 138kDa KCNMA1 protein within the total cellular protein in comparison to exposure to TSA alone (Figure 4.5A; $p<0.05$). This fragment was expressed at a level similar to the TNF samples, indicating that TSA does not affect the effect of TNF on this fragment.

When the cultures were exposed to TSA prior to TNF and blotted with C-terminal antibody the 138kDa KCNMA1 protein was significantly decreased in the total protein samples (Figure 4.5A; $p<0.05$) in comparison to the un-stimulated samples. The 138kDa KCNMA1 protein was significantly decreased within the total cellular protein in comparison to TNF samples (Figure 4.5A; $p<0.05$) indicating that in this order TNF and TSA have a combined effect which is different to either ones individual affect. Within the cytoplasmic fraction the 138kDa KCNMA1 protein was significantly decreased in comparison to TSA samples (Figure 4.5B; $p<0.05$). Indicating that in this order TSA and TNF again have a combined effect which is different to and greater than the effect of each individual stimulus.

Although it is important to understand the mechanism driving the combined effect of TNF and TSA on the KCNMA1 channel it is pertinent to remember that they did not cause any significant change in membrane bound KCNMA1 protein and therefore may not influence the contractility of the cell.

4.4 The CACNA1C Channel

4.4.1 CACNA1C Protein Isoforms are Expressed in Myometrial Cell Cultures and are Unaffected by Either TNF or TSA

This present research sought to determine if TNF or TSA could influence expression of the CACNA1C protein. Total cellular protein was isolated, separated by SDS PAGE and

subsequently western blotted with an antibody directed to the N-terminus of the CACNA1C protein.

Blotting with the N-terminal antibody revealed that the primary myocytes express both the long (240kDa) and short form (190kDa) of the CACNA1C which have been previously documented (Figure 4.6A; Hell, Westenbroek *et al.* 1996). The 240kDa band is representative of the full length CACNA1C protein. The 190kDa band is representative of CACNA1C proteins which have a modified C-terminus (De Jongh, Warner *et al.* 1991). This shorter protein is formed by post-translational proteolytic processing of the C-terminus which occurs *in vivo* and not as an artefact of the protein purification process (De Jongh, Colvin *et al.* 1994). The 240kDa form was approximately two-fold more abundant than the 190kDa form (Figure 4.6A).

When cultures were subsequently exposed to either TNF or TSA, neither had any significant effect on either the CACNA1C protein isoforms or the fragments expressed in comparison to the un-stimulated samples (Figure 4.6A).

4.4.2 Membrane Localisation of the CACNA1C 190kDa Protein Isoform Expressed in Myometrial Cell Cultures is Significantly Increased by TNF

Next it was determined if these compounds had an effect on the localisation of the CACNA1C protein isoforms. Protein was isolated by sub cellular fractionation, separated by SDS PAGE and subsequently western blotted with an antibody directed to the N-terminus of the CACNA1C protein. Blotting with the N-terminal antibody revealed that the long (240kDa) and the short (190kDa) CACNA1C isoforms are located in both the cytoplasmic and membrane fractions (Figure 4.6B, C). This illustrates the importance of sub cellular localisation of the channel proteins as only a portion of the CACNA1C detected in the total cellular protein is localised to the cell membrane and hence will have an effect on cell contractility.

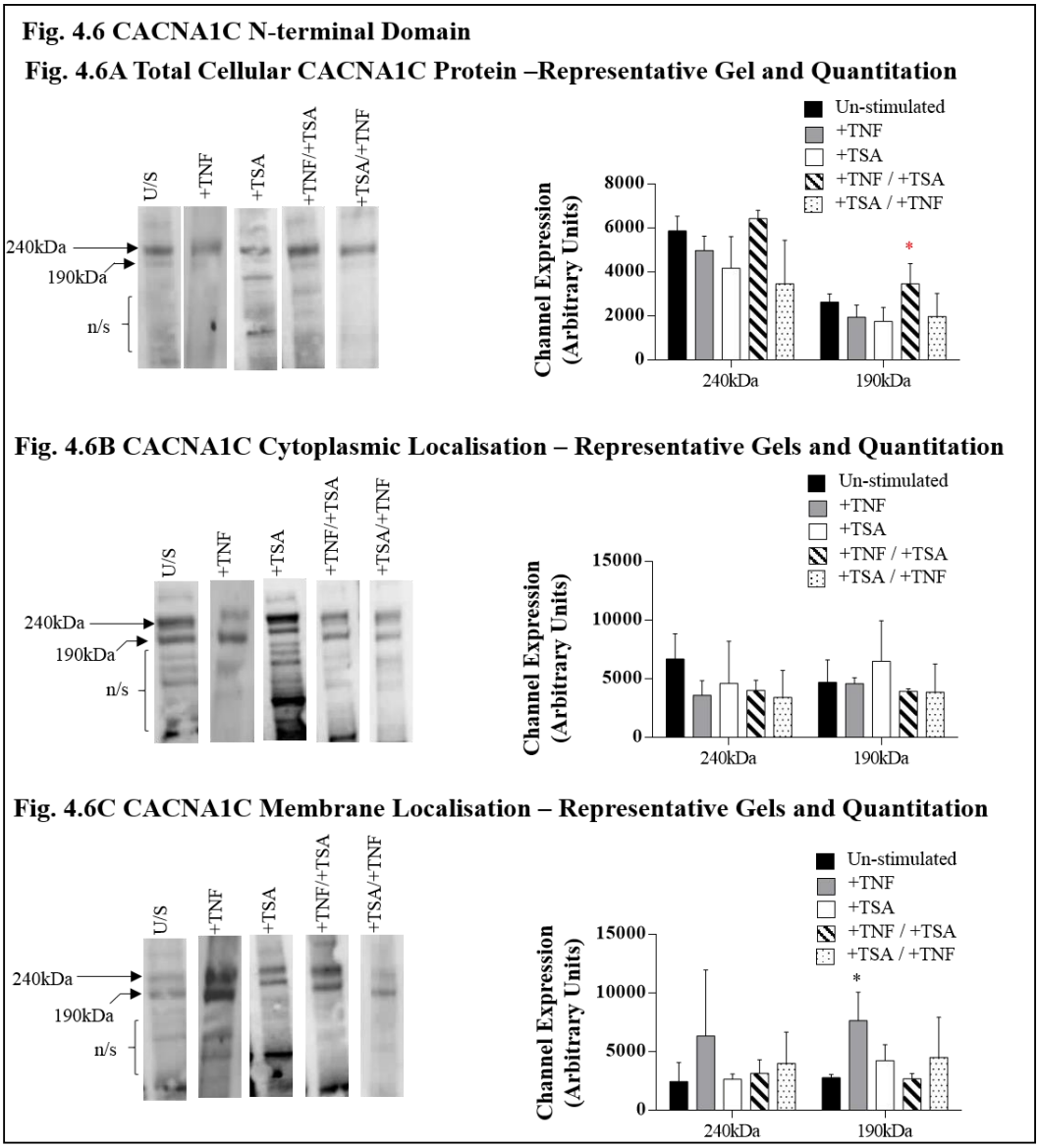


Figure 4.6 The Expression of the CACNA1C Protein in Myometrial Cell Cultures Utilising an N-terminal Antibody

Myometrial myocyte cell cultures were stimulated with 10ng/ml TNF for twenty-four hours, 100ng/ml TSA for twenty-four hours, 10ng/ml TNF for 1 hour followed by 100ng/ml TSA for twenty-four hours (+TNF/+TSA), 100ng/ml TSA for twenty-four hours followed by 10ng/ml TNF for 1 hour (+TSA/+TNF) or left un-stimulated. Protein was extracted, separated by SDS PAGE and western blotted. Manual quantification of the relative band intensities were used to quantify the level of expression and this was expressed as arbitrary units (n = 3). **(A)** Within total cellular protein myometrial cell cultures express two CACNA1C proteins (right panel), +TNF/+TSA caused a significant up regulation of the 190kDa fragment in comparison to TSA ($p<0.05$*). **(B)** Cytoplasmic localisation of the CACNA1C protein (right panel), no treatment had any effect CACNA1C protein expression (left panel) **(C)** Membrane localisation of the CACNA1C protein (right panel), no treatment had any effect CACNA1C protein expression (left panel).

When cultures were subsequently exposed to either TNF or TSA, TNF resulted in a significant increase in the membrane localisation of the short (190kDa) CACNA1C protein isoform in comparison to un-stimulated samples (Figure 4.6C; $p<0.05$). The short form of the CACNA1C lacks the C-terminal tail region, loss of the C-terminus of the

CACNA1C has been shown to result in a 4-6 fold increase in ion conductance in comparison to the full length channel. The presence of this isoform of the CACNA1C would support contraction. TNF did not result in a significant change in the expression or localisation of the longer (240kDa) CACNA1C isoform in comparison to un-stimulated samples (Figure 4.6B, C). TSA had no significant effect on the expression or localisation of either of the two CACNA1C protein isoforms in comparison to un-stimulated samples (Figure 4.6B, C).

4.4.3 Localisation of the CACNA1C Protein Isoforms Expressed in Myometrial Cell Cultures is Unaffected by Combined TSA and TNF Stimulation.

Next it was determined if these compounds had an enhancing or diminishing effect on each other.

When the cultures were exposed to TNF prior to exposure to TSA and then blotted with the N-terminal antibody there was no significant effect on the expression of either of the CACNA1C protein isoforms, protein fragments or their subcellular localisation in comparison to un-stimulated samples (Figure 4.6A, B, C). However, the short (190kDa) CACNA1C protein isoform was significantly increased within the total cellular protein in comparison to TSA (Figure 4.6A; $p<0.05$). As with the KCNMA1 channel, in this order TNF and TSA have a combined effect which is different to and greater than the effect of each individual stimulus.

When the cultures were exposed to TSA prior to TNF and blotted with N-terminal antibody there was again no significant effect on the expression of the CACNA1C channel protein, protein fragments or their subcellular localisation in comparison to un-stimulated samples (Figure 4.6A, B, C).

4.4.4 CACNA1C C-terminal Protein Fragments are Expressed in Myometrial Cell Cultures and are Unaffected by Either TNF or TSA

This present research sought to determine if TNF or TSA could influence expression of the CACNA1C protein. Total cellular protein was isolated, separated by SDS PAGE and

subsequently western blotted with an antibody directed to the C-terminus of the CACNA1C protein.

When western blotting was carried out using the CACNA1C C-terminal antibody it revealed that within the primary myocytes the CACNA1C C-terminal region has been proteolytically processed resulting in the generation of a number of CACNA1C protein fragments (Figure 4.7A). The 48 and 30kDa fragments correspond to those previously documented by Gao *et al.* (Gao, Cuadra *et al.* 2001), however a third previously un-reported 102kDa fragment was also detected (Figure 4.7A). This 102kDa fragment was only detected at a very low level within the total cellular protein, and appears to comprise the entire C-terminal tail, this may have arisen as a result of the extraction process or it may be a novel *in vitro* proteolytic cleavage product.

When cultures were subsequently exposed to either TNF or TSA, neither had any significant effect on either the CACNA1C protein isoforms or the fragments expressed in comparison to the un-stimulated samples (Figure 4.7A).

4.4.5 Localisation of the CACNA1C C-terminal Protein Fragments Expressed in Myometrial Cell Cultures is Unaffected by Either TNF or TSA

Next it was determined if these compounds had an effect on the localisation of the CACNA1C C-terminal protein fragments. Protein was isolated by sub cellular fractionation, separated by SDS PAGE and subsequently western blotted with an antibody directed to the C-terminus of the CACNA1C protein.

Following blotting with the C-terminal antibody the small C-terminal fragments were found in both the cytoplasm and the membrane fractions (Figure 4.7B, C).

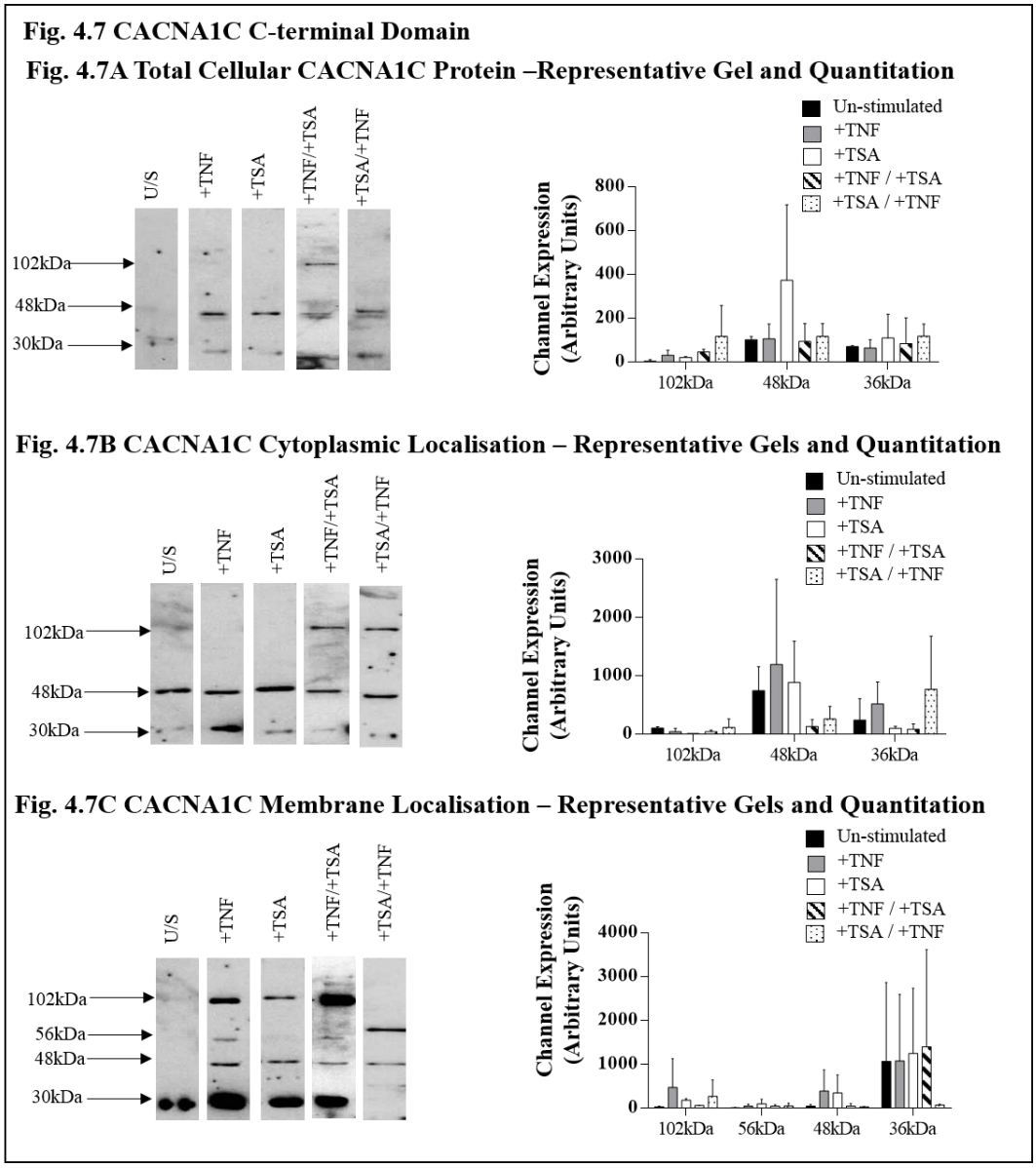


Figure 4.7 The Expression of the CACNA1C Protein in Myometrial Cell Cultures Utilising a C-terminal Antibody

Myometrial myocyte cell cultures were stimulated with 10ng/ml TNF for twenty-four hours, 100ng/ml TSA for twenty-four hours, 10ng/ml TNF for 1 hour followed by 100ng/ml TSA for twenty-four hours (+TNF/+TSA), 100ng/ml TSA for twenty-four hours followed by 10ng/ml TNF for 1 hour (+TSA/+TNF) or left un-stimulated. Protein was extracted, separated by SDS PAGE and western blotted. Manual quantification of the relative band intensities were used to quantify the level of expression and this was expressed as arbitrary units (n = 3). **(A)** Within total cellular protein myometrial cell cultures express a number of CACNA1C proteins fragments (right panel), no treatment had any effect CACNA1C protein expression (left panel). **(B)** Cytoplasmic localisation of the KCNMA1 protein (right panel), no treatment had any effect CACNA1C protein expression (left panel) **(C)** Membrane localisation of the CACNA1C protein (right panel), no treatment had any effect CACNA1C protein expression (left panel).

The cytoplasmic fraction contained the 102, 48 and 30kDa fragments previously observed in the total cell protein blots whilst the membrane contained a 56kDa fragment in addition to the 102, 48 and 30kDa fragments (Figure 4.7C). The significance of this observation is still to be fully explored.

Neither TNF nor TSA had any significant effect on the expression or localisation of any of the small C-terminal fragments in comparison to un-stimulated samples (Figure 4.7B, C). However, the C-terminal fragment blots were inconsistent which may have contributed to the lack of any statistical significance being found between the different groups.

4.4.6 Localisation of the CACNA1C C-terminal Protein Fragments Expressed in Myometrial Cell Cultures is Unaffected by Combined TSA and TNF Stimulation.

Next it was determined if these compounds had an enhancing or diminishing effect on each other.

When the cultures were exposed to TNF and TSA in either order and then blotted with the C-terminal antibody there was no significant effect on the expression of the CACNA1C channel protein, protein fragments or their subcellular localisation in comparison to un-stimulated samples (Figure 4.7A, B, C).

4.5 The CACNA1G Channel

4.5.1 CACNA1G Protein Isoforms are Expressed in Myometrial Cell Cultures and are Unaffected by Either TNF or TSA

This present research sought to determine if TNF or TSA could influence expression of the CACNA1G protein. Total cellular protein was isolated, separated by SDS PAGE and subsequently western blotted. Blotting with the CACNA1G N-terminal antibody revealed that the primary myocytes express both the long (262kDa) and short form (249kDa) of the CACNA1G which have been previously documented (Figure 4.8A; Monteil, Chemin et al. 2000). The 249kDa form utilises exons 25 and 27 whilst the 262kDa form utilises exons 25, 26 and 27. The 249kDa form has the most positive voltage range for steady state inactivation (Monteil, Chemin *et al.* 2000) and so will inactivate more slowly hence it will provide a greater contribution to the influx of calcium into the cell.

When cultures were subsequently exposed to either TNF or TSA, TNF resulted in a small increase in the 249kDa form whilst TSA resulted in a small decrease in the 262kDa form although neither of these reached significance (Figure 4.8A).

4.5.2 Membrane Localisation of the CACNA1G 262kDa Protein Isoform Expressed in Myometrial Cell Cultures is Increased by both TNF and TSA

Next it was determined if these compounds had an effect on the localisation of the CACNA1G protein or protein fragments. Protein was isolated by sub cellular fractionation, separated by SDS PAGE and subsequently western blotted.

Blotting with the CACNA1G antibody revealed that only the 249kDa isoform was located within the cytoplasmic fraction (Figure 4.8B), however, the cytoplasmic protein fraction was also seen to contain a band at around 157kDa and one at around 69kDa (Figure 4.8B).

These smaller molecular mass bands (~157 and ~69kDa) do not equate to any previously reported isoforms of the CACNA1G calcium channel. The most likely explanation is that these bands are as a result of a proteolytic process which occurred during the purification procedure and are therefore not physiologically relevant. This hypothesis is supported by the fact that these small molecular weight bands are only present in the cytoplasmic fraction and the full length protein is completely absent from this fraction. Within the membrane fraction there was no evidence of the 249kDa isoform and the 262kDa isoform was only expressed at a very low level (Figure 4.8C).

When cultures were subsequently exposed to TNF or TSA, neither had any effect on the expression of either the 249kDa isoform of the protein fragments within the cytoplasmic fraction (Figure 4.8B). However, both TNF and TSA increased the amount of the 262kDa isoform within the membrane fraction in comparison to un-stimulated samples although this did not reach significance (Figure 4.8C).

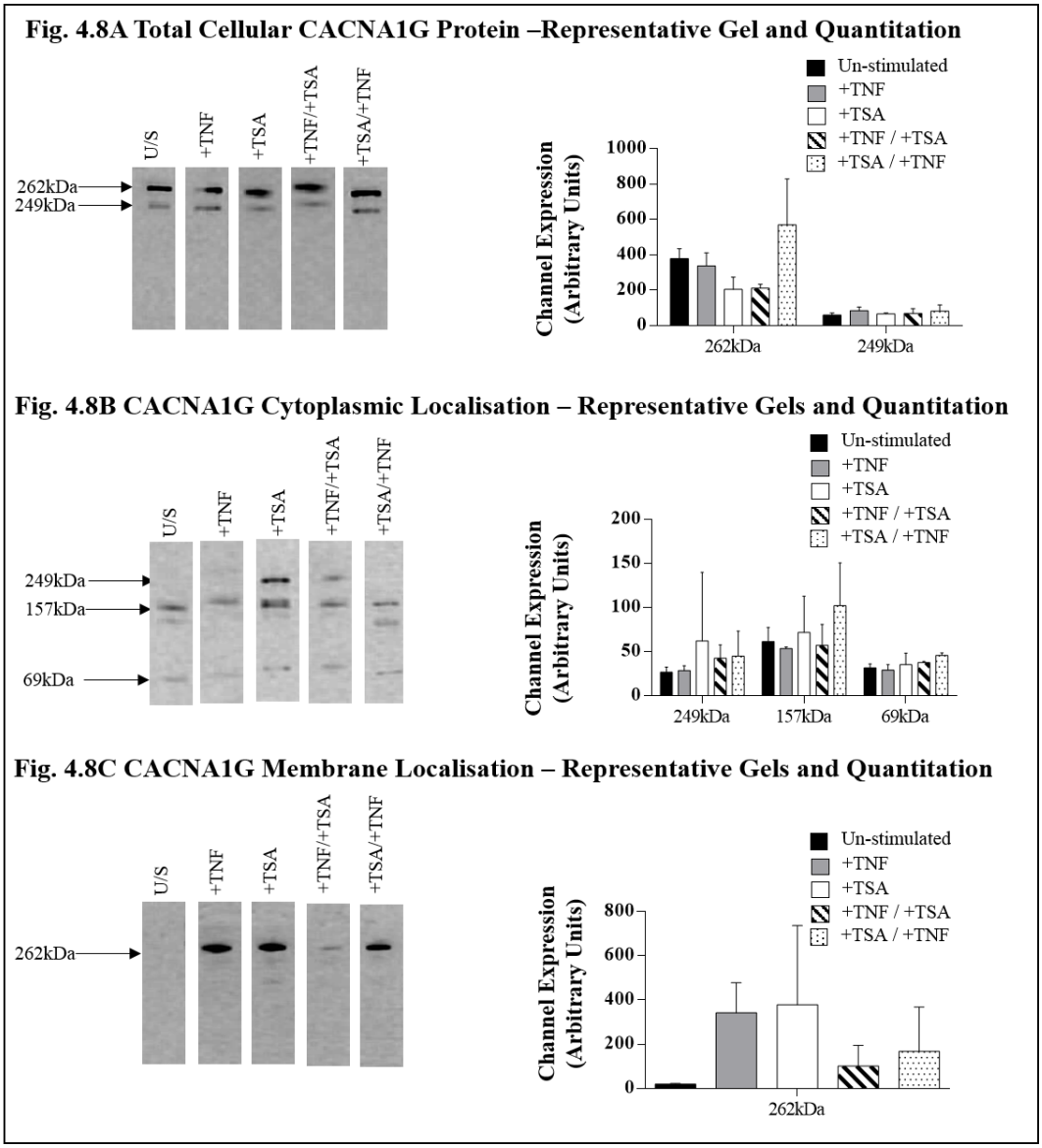


Figure 4.8 The Expression of the CACNA1G Protein in Myometrial Cell Cultures Utilising an N-terminal Antibody

Myometrial myocyte cell cultures were stimulated with 10ng/ml TNF for twenty-four hours, 100ng/ml TSA for twenty-four hours, 10ng/ml TNF for 1 hour followed by 100ng/ml TSA for twenty-four hours (+TNF/+TSA), 100ng/ml TSA for twenty-four hours followed by 10ng/ml TNF for 1 hour (+TSA/+TNF) or left un-stimulated. Protein was extracted, separated by SDS PAGE and western blotted. Manual quantification of the relative band intensities were used to quantify the level of expression and this was expressed as arbitrary units (n = 3). **(A)** Within total cellular protein myometrial cell cultures express a two CACNA1G proteins (right panel), no treatment had any effect CACNA1G protein expression (left panel). **(B)** Cytoplasmic localisation of the CACNA1G protein (right panel), within this fraction two addition protein fragment were observed (right panel). No treatment had any effect CACNA1G protein expression (left panel) **(C)** Membrane localisation of the CACNA1G protein (right panel), no treatment had any effect CACNA1C protein expression (left panel).

4.5.3 Localisation of the CACNA1G Protein Isoforms Expressed in Myometrial Cell Cultures is Unaffected by Combined TSA and TNF Stimulation.

It was then determined whether these compounds had an enhancing or diminishing effect on each other. When the cultures were exposed to TNF and TSA in either order and then blotted with the CACNA1G antibody there was no significant effect on the expression of the CACNA1G channel protein isoforms or their subcellular localisation in comparison to un-stimulated samples (Figure 4.8A, B, C).

4.6 Nuclear Localisation

4.6.1 The CACNA1C Protein is Found Localised Within the Nucleus in Myometrial Cell Cultures

Previous research has demonstrated that once cleaved, the CACNA1C C-terminal fragment can either co-localise with the rest of the channel (Hell, Westenbroek *et al.* 1996, Gerhardstein, Gao *et al.* 2000, Hulme, Konoki *et al.* 2005, Hulme, Yarov-Yarovoy *et al.* 2006, Schroder, Byse *et al.* 2009, Satin, Schroder *et al.* 2011) or can move into the nucleus (Gomez-Ospina, Tsuruta *et al.* 2006, Schroder, Byse *et al.* 2009, Satin, Schroder *et al.* 2011). Within the nucleus the C-terminal fragment of the CACNA1C has been shown to act as a transcription factor. It was shown to bind to its own promoter and down regulate transcription (Schroder, Byse *et al.* 2009, Satin, Schroder *et al.* 2011). It has also been shown to regulate the expression of a number of genes including connexin 3.1 in neurons; the Na/Ca exchanger, which works co-ordinately with the calcium channel to maintain cellular calcium (Schroder, Byse *et al.* 2009) and the potassium channel KCNN3. To determine if the C-terminus of the CACNA1C enters the nucleus within the primary myocyte cultures soluble nuclear and chromatin bound nuclear protein was isolated by sub-cellular fractionation, separated by SDS PAGE and subsequently western blotted. After blotting with the C-terminal CACNA1C antibody a 30kDa bind was observed within the soluble nuclear fraction (Figure 4.10A), however no band was observed in the chromatin bound nuclear fraction (Figure 4.10B).

When cultures were subsequently exposed to either TNF or TSA, a 30kDa CACNA1C protein band was observed in the soluble nuclear protein fraction (Figure 4.10A), again there was no band observed in the chromatin bound nuclear fraction (Figure 4.10B). No

statistical analysis was carried out on these results as the gels were loaded with the same volume of protein and not the same concentration due to the low concentration of protein extracted in these fractions.

Although the C-terminal was not localised in the chromatin nuclear fraction in this study, as it was found within the soluble nuclear fraction it is possible that it still has role regulating transcription in these cells. Although it is outside the bounds of this thesis the role of the CACNA1C C-terminal transcription factor within primary myocytes is worthy of further investigation.

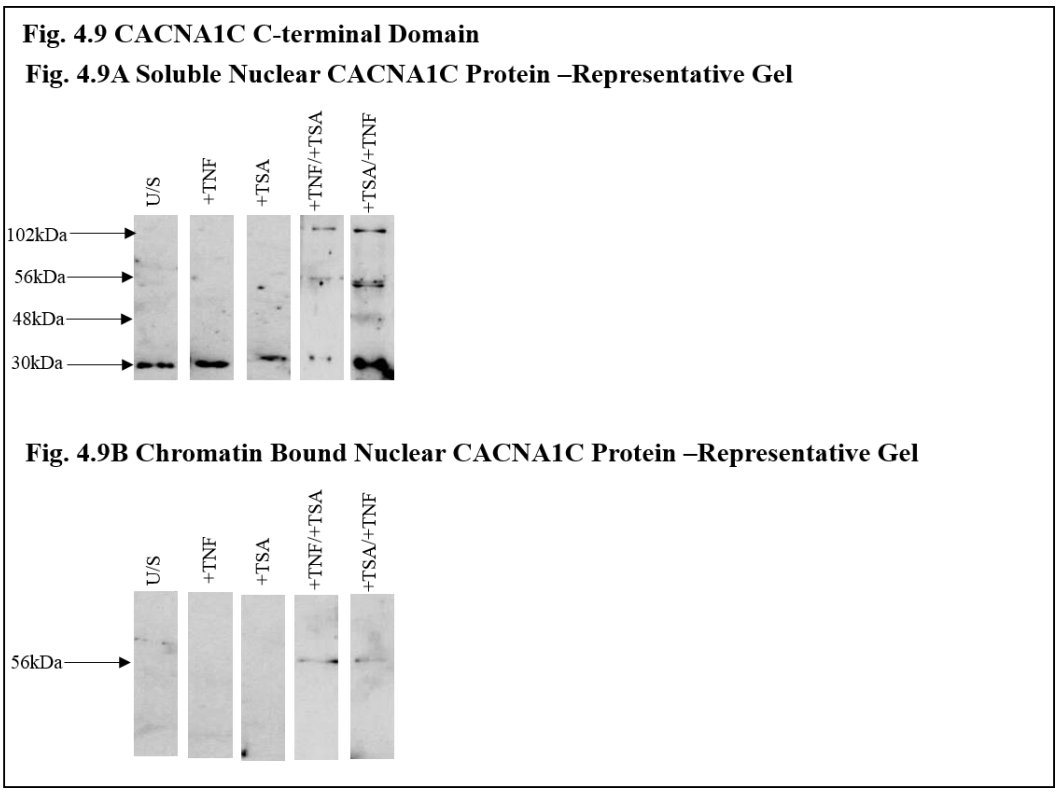


Figure 4.9 Nuclear Localisation of the CACNA1C Protein in Myometrial Cell Cultures Utilising a C-terminal Antibody

Myometrial myocyte cell cultures were stimulated with 10ng/ml TNF for 1 hour, 100ng/ml TSA for twenty-four hours, or left un-stimulated. Protein was extracted, separated by SDS PAGE and western blotted. Manual quantification of the relative band intensities were used to quantify the level of expression and this was expressed as arbitrary units (n = 3). **(A)** The CACNA1C was detected within the soluble nuclear protein fraction **(B)** The CACNA1C was not detected within the chromatin bound nuclear protein fraction.

4.6.2 The KCNMA1 Channel Protein is Found Localised Within the Nucleus in Myometrial Cell Cultures.

In Chapter 3, following immunocytochemistry staining of the primary myocytes nuclear localisation of the KCNMA1 channel was observed. This was a novel finding, therefore it was important to rule out the possibility that this nuclear localisation was a staining artefact. To do this, soluble nuclear and chromatin-bound nuclear protein was isolated by sub-cellular fractionation, separated by SDS PAGE and subsequently western blotted. After blotting with the KCNMA1 C-terminal antibody in the un-stimulated samples there was no KCNMA1 protein found within the soluble nuclear protein fraction (Figure 4.9A), however, within the chromatin bound nuclear fraction there was a band for the 138kDa KCNMA1 protein (Figure 4.9B).

When cultures were subsequently exposed to either TNF or TSA, both resulted in the appearance of a 138kDa and a 65kDa KCNMA1 protein band within the soluble nuclear fraction (Figure 4.9A). A faint 138kDa KCNMA1 band was observed in the chromatin bound nuclear fraction following exposure to TNF, however no bands were observed in this fraction after exposure to TSA (Figure 4.9B). No statistical analysis was carried out on these results as the gels were loaded with the same volume of protein and not the same concentration due to the low concentration of protein extracted in these fractions.

Although these findings support the immunocytochemistry observations, it is important to remember that the bands observed could be a result of protein carry over during the sub-cellular fractionation protocol. The putative role of the KCNMA1 channel within the nucleus is still to be fully explored.

Fig. 4.10 KCNMA1 C-terminal Domain

Fig. 4.10A Soluble Nuclear KCNMA1 Channel Protein –Representative Gel

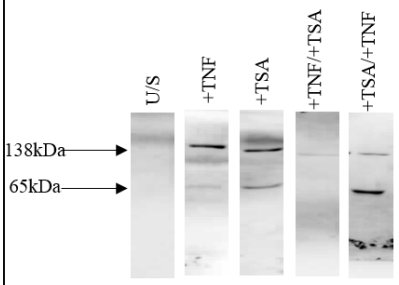


Fig. 4.10B Chromatin bound Nuclear KCNMA1 Channel Protein –Representative Gel

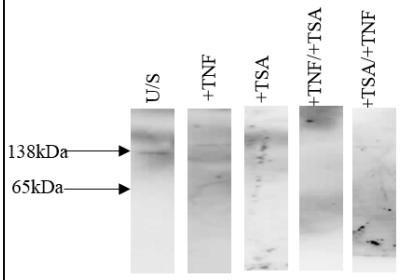


Figure 4.9 Nuclear Localisation of the KCNMA1 Protein in Myometrial Cell Cultures Utilising a C-terminal Antibody

Myometrial myocyte cell cultures were stimulated with 10ng/ml TNF for 1 hour, 100ng/ml TSA for twenty-four hours, or left un-stimulated. Protein was extracted, separated by SDS PAGE and western blotted. Manual quantification of the relative band intensities were used to quantify the level of expression and this was expressed as arbitrary units (n = 3). **(A)** The KCNMA1 channel was detected within the soluble nuclear protein fraction **(B)** The KCNMA1 channel was detected within the chromatin bound nuclear protein fraction

4.7 Discussion

4.7.1 The KCNMA1 Channel Protein Antibodies

Firstly, it is necessary to address the differences seen in the results from the two different KCNMA1 antibodies. The KCNMA1 N-terminal antibody was raised against a synthetic peptide from the N-terminal of the Human KCNMA1 channel conjugated to an immunogenic carrier protein, it was supplied as whole serum, not affinity purified and it had only been tested in western blot against the recombinant protein used as an immunogen. Although these factors would seem to make it a poor choice of antibody for this purpose, it was chosen as the majority of other KCNMA1 N-terminal antibodies were raised in goat. The issue with using primary antibodies raised in goat for western blotting is finding an appropriate blocking solution because the anti-goat secondary will cross-react with both BSA and milk-containing blocking buffers giving a very high non-specific background signal (Buchwalow, Samoilova *et al.* 2011). The original KCNMA1 N-

terminal antibody was raised in goat and despite optimisation it was not possible to acquire a suitable signal:noise ratio using a variety of blocking buffers including BSA, Milk, non-protein block or serum block at any of the primary and secondary antibody concentration combinations investigated. Therefore, a pragmatic decision was taken: although the KCNMA1 N-terminal antibody used was not ideal it was the better choice. The antibody itself may therefore have contributed to the differences seen in comparison to the C-terminal antibody.

There are also intrinsic differences between the protein structures in the region of the binding sites for the N- and C-terminal KCNMA1 channel proteins. The N-terminal antibody binds between amino acids 100 and 150 of the protein, this region encompasses part of the intracellular loop between the S0 and S1 segments, the transmembrane S1 segment and also part of the intracellular loop between segments S1 and S2 while the C-terminal antibody binds at the very end of the intracellular C-terminal tail region. The N-terminal antibody binds in a region composed of transmembrane helix structures and intracellular linking loops while the C-terminal is 848 amino acids in length and is composed of intracellular helix and beta strand structures.

The N-terminal region and the S0 transmembrane spanning domain are responsible for coupling of the β-subunit (Wallner, Meera *et al.* 1996, Morera, Alioua *et al.* 2012). The β-subunit increases the calcium and voltage sensitivity of the KCNMA1 channel and hence supports quiescence. Korovkina *et al.* identified and characterised a KCNMA1 variant in which a 132bp exon is inserted between exons 1 and 2 resulting in an additional 44aa being inserted in the linking loop between S0 and S1. The inserted amino acids contain two consensus sites for protein phosphorylation and also a myristylation site (Korovkina, Fergus *et al.* 2001, Korovkina, Brainard *et al.* 2006). KCNMA1 channel with this insert have been shown to be proteolytically cleaved, when cleaved the N-terminus is located in the membrane and the C-terminus localises to the sarcoplasmic reticulum. The binding site for the N-terminal antibody is in the vicinity of this proteolytic cleavage and therefore if the channel has been cleaved this may destroy the antibody binding site (Figure 4.11).

All of the above are plausible explanations for why the N-Terminal antibody worked less well in the western blot but ultimately the most probable explanation is that as the

antibody recognised a linear peptide epitope i.e. a synthetic peptide, a harsher denaturing lysis buffer such as RIPA buffer would have been more appropriate. This would have ensured that the antibody recognised the peptide more readily and was more able to bind. The less harsh lysis buffer which was used would have resulted in a decrease in the availability of a suitable epitope for the antibody and hence a reduced signal. This then combined with the non-specificity of a whole-serum antibody gave blots with low signal:noise ratios which were difficult to interpret.

4.7.2 The KCNMA1 Channel Protein Fragments

Due to the low signal:noise ratios achieved in the N-terminal western blots it is necessary to treat the data generated from these with caution until it can be validated. Various fragments were detected by the N-terminal antibody that were not detected when the C-terminal antibody was used, indicating that these fragments were lacking the C-terminal region. Initially these fragments were dismissed as not physiologically relevant as they had been identified previously by Knaus *et al.* and were determined to be by-products of proteolytic cleavage which has occurred as a result of the purification process (Knaus, Eberhart *et al.* 1995).

Utilising the knowledge that it is the C-Terminal region that is absent it is possible to approximate the location of the cleavage sites for these fragments and interestingly the cleavage sites for all three fragments (50, 65 and 90kDa) occur in regions of known splice variants (Figure 4.11).

Fig. 4.11 MaxiK Channel Protein

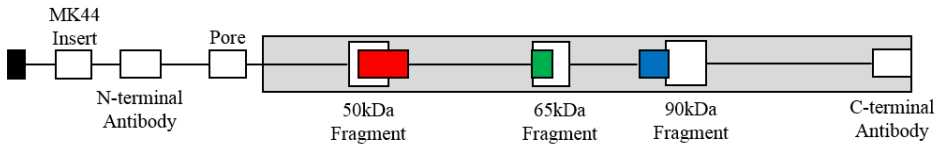


Figure 4.10 Diagrammatic Representation of the KCNMA1 protein
Details the location of key features including the channel pore, splice sites (M2-Red; M3-Green; M5-Blue). The C-terminus is highlighted with a grey box and the N-terminus with a black box. The putative cleavage sites for the generation of the protein fragments are highlighted by the white boxes. The antibody binding sites are detailed as is the Mk44 insertion.

The 90kDa fragment is cleaved in the region of splice site M5 (Figure 4.11), this site is located in the linking loop between S9 and S10 in close proximity to the 'calcium bowl'.

Splice site M5 involves the insertion or deletion of exon 29 and results in KCNMA1 channels with an increased activation rate (Tseng-Crank, Foster *et al.* 1994, Yan, Olsen *et al.* 2008). In a separate study to identify where the calcium sensitivity of the KCNMA1 channel was located Schreiber *et al.* demonstrated that abolishing the sensitivity of the calcium bowl resulted in KCNMA1 channels with increased activation rates (Schreiber, Yuan *et al.* 1999). Taken together with the protein and splice variant data in this study, it would indicate that the insertion of exon 29 could result in the introduction of a cleavage site which then results in the cleavage of the C-terminal tail region effectively removing the calcium bowl from the core of the channel and abolishing the channels calcium sensitivity resulting in a channel with increased activation rates.

The 65kDa fragment is cleaved in the region of splice site M3 (Figure 4.11), this site is located in the linking loop between S8 and S9, the effects of this splice variant have not yet been fully identified. The splice site involves differential utilisation of exon 19, at the protein level this results in the insertion of 40aa (the utilisation of the entire of exon 19), the insertion of 4aa (the 3' truncation of exon 19) and no amino acids inserted (the skipping of exon 19). Tseng-Crank demonstrated that the insertion of the 4aa resulted in a KCNMA1 channel with a 3- to 4-fold decrease in calcium sensitivity (Tseng-Crank, Foster *et al.* 1994). These same 4aa are at the start of the 40aa insert but the effect this insert had on calcium sensitivity was not examined. Three of the four inserted amino acids are positively charged and Tseng suggested that the decrease in calcium sensitivity was either because this positive charge was interacting with the calcium binding site or because the inserted amino acids were causing conformational changes in the protein structure (Tseng-Crank, Foster *et al.* 1994). Combining the splice variant data, which shows that the complete exon 19 is utilised in these primary myocytes, with the protein data reported here suggests a third possibility: the utilisation of the complete exon 19 introduces a cleavage site and that the calcium sensing C-terminal tail can then be cleaved from the channel. This would have the effect of reducing the calcium sensitivity of the channel.

The 50kDa fragment is cleaved in the region of splice site M2 (Figure 4.11), this site involves the mutually exclusive use of exons 10 and 11 or exons 11 and 12. This can result in either truncated non-functional channels or functional channels with strongly altered gating kinetics (Soom, Gessner *et al.* 2008). Interestingly, if the location of the C-

terminal antibody is utilised to approximate the cleavage site for the 65kDa fragment detected by this antibody, the cleavage for this fragment occurs in a similar location to the cleavage site of the 50kDa fragment: this may indicate that the two fragments complement each other. The splice variant data have shown that the primary myocytes express exon 11 and 12, and so the C-terminally truncated 50kDa fragment may be generated as a result of proteolytic cleavage of the protein at a cleavage site introduced via the utilisation of exons 11 and 12. The loss of the C-terminal tail region would alter the gating kinetics of the channel which has been reported as an effect of alternative splicing in this region.

To determine if any of these scenarios are physioloigially relevant it would be necessary to determine the structure of the channel *in vivo.* It would also be pertinant to undertake channel activity recordings in order to determine the specific effect on channel gating.

4.7.3 Protein Fragments of the CACNA1C Channel

The N-terminal antibody detected two forms of the CACNA1C channel on the western blots, the first at 240kDa is representative of the full length channel and the second at 190kDa is representative of a CACNA1C channel which has undergone post-translational modification were the C-terminus has been proteolytically cleaved. The C-terminal antibody detected four different fragments.

The 190kDa fragment detected by the N-terminal antibody, has been reported to lack a portion of the C-terminal tail of the channel and this cleavage occurs in a known splicing region (Figure 4.12). C-terminal truncation of the CACNA1C has been described in cardiac cells (Tseng-Crank, Foster *et al.* 1994, Satin and Schroder 2009), brain cells (De Jongh, Colvin *et al.* 1994) and skeletal muscle (Hulme, Konoki *et al.* 2005). This cleavage has been shown to occur between amino acids 1685 - 1699 of the CACNA1C (De Jongh, Warner *et al.* 1991) close to a calpain cleavage site. Indirect evidence suggests that a calpain-like protease is responsible for the proteolytic processing of the channel. De Jongh *et al.* demonstrated that calpain I is capable of cleaving a 37kDa fragment from the C-terminus of the CACNA1C (De Jongh, Colvin *et al.* 1994). This would correlate well with the smallest fragment detected by the C-terminal antibody and as such this small fragment and the 190kDa fragment may actually be generated as a result of a single

cleavage event. Hell *et al.* went on to show that this cleavage was induced by an influx of calcium and that this cleavage can be blocked by calpain inhibitors (Hell, Westenbroek *et al.* 1996).

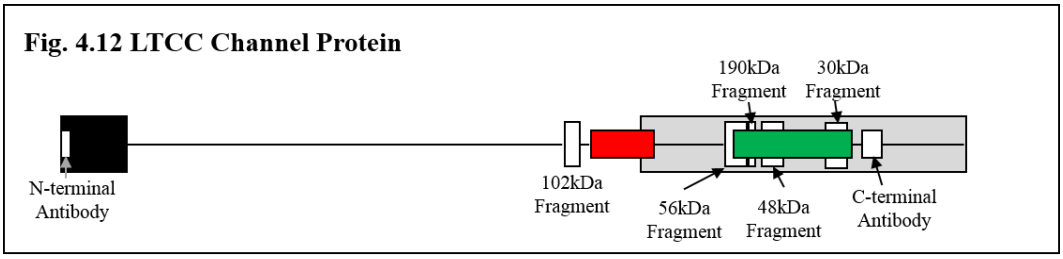


Figure 4.11 Diagrammatic Representation of the CACNA1C Protein
Details the location of key features including, splice sites (L11-Red; L12-Green). The C-terminus is highlighted with a grey box and the N-terminus with a black box. The putative cleavage sites for the generation of the protein fragments are highlighted by the white boxes. The antibody binding sites are detailed.

Reports on the role of the C-terminus and hence the effects of cleaving the C-terminus initially appear to be conflicting. Firstly, it has been shown that cleavage of the C-terminus removes the major site of cAMP dependant phosphorylation from the body of the channel – ser1928 (Gao, Yatani *et al.* 1997). Phosphorylation of this ser-1928 residue has been shown to result in up-regulation of the channel (Gao, Yatani *et al.* 1997). This evidence would suggest then, that cleavage of the C-terminus should result in down regulation of the channel. In fact, however, the proteolytically cleaved CACNA1C calcium channel has been shown to have a 4- to 6-fold higher ion conductance than the full length channel (Wei, Neely *et al.* 1994). The C-terminally cleaved fragment has also been shown to non-covalently associate with the body of the channel on the membrane and exert an inhibitory effect (Gao, Cuadra *et al.* 2001). Wei *et al.* suggested that the C-terminal tail folded over changing the proteins structure and inhibiting the activity of the channel (Wei, Neely *et al.* 1994). If the phosphorylation of ser-1928 resulted in the C-terminus being unable to fold round as Wei suggests then this phosphorylation would, in effect, have the same effect as removal of the C-terminus.

Splicing may be involved in modulating the role of the C-terminus as there are a number of splice sites in the C-terminus which have been shown to modulate the gating properties of the CACNA1C. Splice sites L11 and L12 involve in the inclusion of additional nucleotides and/or the utilisation of an alternative exon and both are in the vicinity of the putative cleavage site (aa1685-1699) and ser-1928 and so it is possible that these splice

variants may either remove the phosphorylation site or introduce a cleavage site. Splice site 11 involves exon 40 which encompasses the putative cleavage site and splice site 12 involves exon 44 which encompasses ser-1928 (Figure 4.12). The splice variant analysis showed that the myocytes express exon 40 with no addition or deletions and so this may contain the cleavage site responsible for the truncation of the CACNA1C.

As with the KCNMA1 channel it would be necessary to determine if these splice sites did indeed have the effects suggested and to undertake channel activity recordings in order to determine the specific effect on channel gating..

4.7.4 The CACNA1G Channel Protein

There were two main protein forms for the CACNA1G channel evident from the western blots, the first at ~262kDa and the second at ~249kDa, this range in protein size of the CACNA1G has been reported previously (Yunker, Sharp *et al.* 2003, Bertolesi, Walia Da Silva *et al.* 2006). Two forms of the CACNA1G were defined by Monteil *et al.*, a short version termed CACNA1G $\alpha_{1G\text{-}b}$ and a longer version termed CACNA1G $\alpha_{1G\text{-}a.}$ These two forms were shown to contain distinct III-IV loops, $\alpha_{1G\text{-}b}$ is formed utilising an alternative 5' splice donor site in exon 25 combined with the acceptor site on exon 27 (Monteil, Chemin *et al.* 2000). The splice variant analysis revealed that the primary myocytes express exons 25 and 26 this would account for the longer form of the CACNA1G. A second splice site which could also account for the detection of a smaller CACNA1G protein is splice site 9 with can include the deletion of exons 34 and 35 (Ernst and Noebels 2009; Figure 4.13), this splice site was not included in the splice variant analysis and so it is not possible to determine if these exons are present or not.

There were a further two smaller fragments of the CACNA1G also detected. The smallest of these (69kDa) occurs within splice site T1 splicing in this region has been shown to result in the modification of protein trafficking (Figure 4.13; Shcheglovitov, Vitko *et al.* 2008). The second fragment (157kDa) does not occur in or near a known splice site and so this fragment may be caused due to the protein extraction techniques and hence is physiologically irrelevant or it may be a novel finding (Figure 4.13). As with the KCNMA1 and CACNA1C further work is need to fully characterise these channel isoforms and fragments.

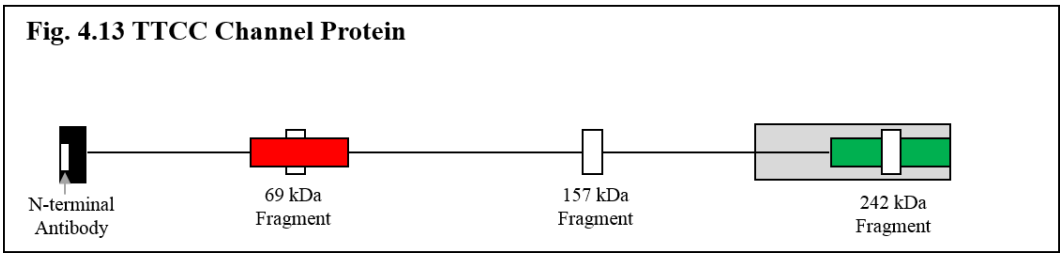


Figure 4.12 Diagrammatic Representation of the CACNA1G Protein
Details the location of key features including splice sites (T1-Red; T9-Green). The C-terminus is highlighted with a grey box and the N-terminus with a black box. The putative cleavage sites for the generation of the protein fragments are highlighted by the white boxes. The antibody binding site is detailed.

4.7.5 The Role of the KCNMA1, CACNA1C and CACNA1G Channels in Myometrial Contractility.

In the un-stimulated samples, which are representative of the end of pregnancy prior to the initiation of contractions, the data shows that both full length and truncated KCNMA1 channel forms are present in both the cytoplasm and inserted into the membrane, full length and post translational modified CACNA1Cs are present again in the cytoplasm and inserted in the membrane, with the cytoplasmic fraction containing more channel than the membrane and full length CACNA1G is present in the whole cell lysis but is not detectable localised to the membrane. This demonstrates that, at this juncture, the myometrial myocytes express channels which have decreased calcium sensitivity (truncated KCNMA1 channel forms), and increased ion conductance (truncated CACNA1C) meaning when the CACNA1C is triggered to open the membrane will depolarise more rapidly and the KCNMA1 channel will respond less rapidly and so the cell will repolarise more slowly tipping the fine balance of the cell in favour of contraction.

At the initiation of labour the myometrium is subject to an influx of cytokines including TNF. Therefore, TNF stimulated cells may serve as a model of this time point. In the primary cells after TNF treatment, full length KCNMA1 channel protein was again seen in both the cytoplasmic and membrane fractions but the level was unchanged from the un-stimulated cultures. Membrane localisation of the 50kDa fragment which represents KCNMA1 channels with decreased calcium sensitivity is increased after TNF treatment. Post-translational modified CACNA1C was also present in both fractions and membrane localisation of this protein was increased after TNF treatment. Finally full length

CACNA1G was detected localised to the membrane after TNF treatment. Demonstrating that the influx of cytokines, specifically TNF, causes a mobilisation of the post-translationally modified CACNA1C and CACNA1G proteins from the cytoplasm to the membrane whilst promoting the membrane expression of the less responsive 50kDa KCNMA1 channel fragment and hence increases the contractility of the cell.

TSA, is a broad spectrum lysine deactylase inhibitor which has been seen to cause relaxation in myometrial strips, therefore, TSA-stimulated cells promote a relaxed myometrial phenotype. In the primary cells, after TSA treatment, there was an increase in the full length KCNMA1 channel protein although this appears to be restricted to the cytoplasmic fraction. A decrease in full length LTCC was also observed but this was not significant. Visually this decrease in CACNA1C expression appears to be focused in the membrane fraction although again this was not significant. Finally the TTCC was again present localised to the membrane. This demonstrates that TSA may act by increasing transcription of the KCNMA1 channel and by supporting the removal of the CACNA1C from the membrane. Importantly TSA seems to be working with slower kinetics than seen with TNF.

If histone deacetylase inhibitors are to be developed as tocolytic agents it is important to determine the combined effects of TNF and TSA. This will inform the decision of whether the tocolytic is more effective administered prior to the cytokine influx or after it.

As TSA has been shown to cause relaxation of the primary cultures it is perhaps unsurprising to see that it also causes an increase in KCNMA1 channel protein. However this increase in channel protein appears to be confined to the cytoplasmic fraction and not the membrane fraction and so would not be expected to contribute to the excitation of the membrane.

It is interesting to note that when TNF is added, either before or after TSA, the TSA-associated increase in KCNMA1 channel protein is not seen indicating that TNF can abrogate the effects of TSA on the expression of this protein. TSA has been shown to induce ubiquitin-dependent degredation of proteins via the 26s proteasome pathway (Caron, Boyault *et al.* 2005, Alao, Stavropoulou *et al.* 2006, Kong, Lin *et al.* 2006). Research by Dennis *et al.* demonstrated the disruption of proteosome function can inhibit

progesterone receptor-dependant transcription in a delayed manner. This inhibition was linked to a decrease in promoter histone acetylation, however treatment with TSA was unable to restore progesterone mediated transcription (Dennis, Lonard *et al.* 2005). Progesterone has been shown to cause an increase in the expression of the KCNMA1 STREX exon (Holdiman, Fergus *et al.* 2002, Zhu, Eghbali *et al.* 2005) and TNF has been shown to to up-regulate progesterone receptor A (Jiang, Guo *et al.* 2012). Progesterone receptor A has been shown to repress transcription (Giangrande, Kimbrel *et al.* 2000, Condon, Hardy *et al.* 2006) and therefore it is possible that in the case of KCNMA1 TSA is unable to reverse the effect of TNF on the progesterone receptor-dependent transcription of the channel.

The effect of TSA and TNF together on the KCNMA1 channel can be different than that of TNF or TSA on their own. For example within the cytoplasmic fraction the mean intensity of the KCNMA1 138kDa band is increased in the TNF and TSA stimulated groups compared to the un-stimulated group. The intensity of this band then drops to below the intensity of the un-stimulated band in both the +TNF/+TSA and +TSA/+TNF groups. This could be because the action of TSA represses the transcription of IκBα (Wu, Starzinski-Powitz *et al.* 2010) and so the usual negative feedback loops of IκBα and A20 may no longer function and inhibit the cascade caused by TNF. The effect is therefore enhanced when the TNF cascade is triggered then the negative feedback is blocked as is the case with the +TNF/+TSA group.

4.8 Conclusion

This data has demonstrated that there are multiple isoforms of the KCNMA1, CACNA1C and CACNA1G expressed within the primary myocyte cultures. These isoforms could be generated either by splicing of the RNA or proteolytic processing of the protein. The presence of various protein fragments for both the KCNMA1 and CACNA1C protein indicate that these proteins have been cleaved. This cleavage may have been made possible by the utilisation of different splice variants in the generation of the RNA. The fragments generated by these cleavage events have been shown to be capable of regulating channel function and as such have a role in regulating the contractility of the cell. Another way in which channel function can be modulated is via the proteins it associates with on the cell membrane. The different isoforms and fragments of the

KCNMA1 and LTCC reported in this chapter may have role in the selection and or recruitment of other proteins which in turn may enhance or repress the action of the channel. The next step therefore is to uncover what proteins these channels associate with and whether TNF or TSA have an effect on this association, this will be the focus of the following chapter.

Chapter 5: Co-Localisation of the KCNMA1 and CACNA1C Proteins with Caveolin-1, β_2 Adrenergic Receptor and Gαs in Cultured Myometrial Cells Results and Discussion

5.1 Introduction

In Chapter 4 it was shown that TNF causes mobilisation of both the CACNA1C and CACNA1G proteins from the cytoplasm to the membrane whilst promoting the membrane localisation of the C-terminally truncated 50kDa KCNMA1 channel fragment and in this way may increase the contractility of the cell. Moreover, TSA caused a small decrease in the CACNA1C localised to the membrane.

The cell membrane consists of approximately 50% protein. As well as these proteins the membrane also contains small invaginations called caveolae. Caveolae are flask shaped structures which are rich in proteins and lipids, they have a role in the regulation of channels and calcium signalling (Parton and Simons 2007).

Previous research has demonstrated that caveolae can facilitate the localisation of key calcium handling proteins, and has directly linked the β_2AR to both the CACNA1C (Davare, Avdonin *et al.* 2001, Liu, Shi *et al.* 2004) the KCNMA1 channel (Liu, Shi *et al.* 2004) and both channels together (Liu, Shi *et al.* 2004). This macromolecular complex is thought to also contain a guanosine-5'-triphosphate (GTP)-binding protein complex and may also involve actin filaments (Chanrachakul, Broughton Pipkin *et al.* 2004, Brainard, Miller *et al.* 2005, Chanrachakul 2006). This complex would enable specific local regulation of both channels. It has not been elucidated whether this complex exists within the caveolae (Figure 5.1) or whether this is a separate process by which the two channels can be brought into close proximity.

Therefore, the next step was to understand the membrane microenvironment within which the channels are functioning.

Using co-immunoprecipitation and western blotting this chapter seeks to determine what, if any protein complexes containing the KCNMA1 and CACNA1C are present in the myometrium and if these complexes exist within caveolae. Further to this, the possible effect of TNF and TSA on any complexes discovered was investigated

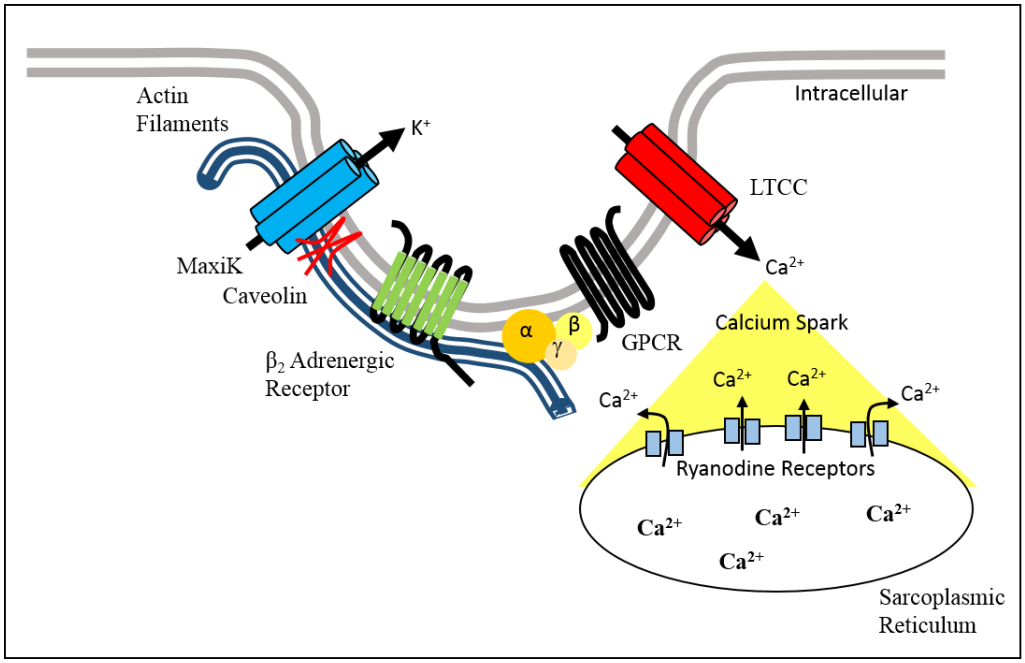


Figure 5.1 Diagrammatic Representation of the Putative Caveolae Microdomain Housing Calcium-handling Proteins
CACNA1C – CACNA1C calcium channel; GPCR – G protein coupled receptor; Ca2+ - Calcium ions; α – G protein α subunit; β – G protein β subunit; γ – G protein γ subunit.

5.2 Optimisation of Protein and Antibody Concentrations for Western Blotting

As in Chapter 4 a dot blot was utilised to optimise the protein and antibody concentrations for use in western blotting. Different quantities of protein (5µg, 10µg and 50µg) were applied directly to a nitrocellulose membrane (Geneflow, Staffs) as dots, then incubated with different dilutions of primary antibody (1:500, 1:1000 and 1:2000). Secondary antibody was then added and the blots developed using Westar Supernova (Geneflow, Staffs) detection reagents. At least one combination for each antibody produced clear dots on the dots blots (Figure 5.2)

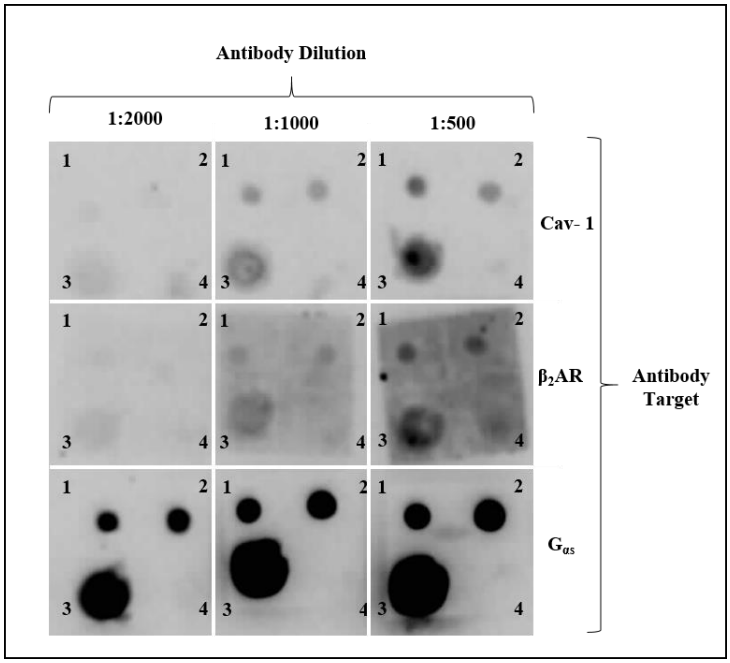


Figure 5.2 Dot Blot Optimisation
The antibody target and antibody dilutions are detailed in the figure. The numbers 1, 2, 3, 4 related to the amount of protein added in that dot; 1 - 5µg; 2 - 10µg; 3 - 50µg and 4 – negative control (no protein). The lack of dot in the negative control of each blot and the lack of background on the majority of blots show the block is effective and that each antibody is working. The dark spots indicate the antibodies are detecting protein. Blots were developed with WestStar Supernova ECL reagent.

5.3 Analysis of Data

After manual band quantification of the western blot images, analysis was performed as follows. Within each sample set, the un-stimulated sample was quantified as 100%, the stimulated samples for the matching western blots were then quantified as a percentage of the un-stimulated sample. For the co-immunoprecipitation experiments the value ascribed to non-specific binding of IgG was subtracted from the specific binding value. That value was then expressed as a percentage of the input fraction with the input fraction intensity being taken as 100%. All experiments were repeated three times and the results are expressed as the mean ± SD. All data analyses were conducted on GraphPad Prism version 6 (GraphPad Software, San Diego, California), where a one way ANOVA with Dunett's post-test was performed to compare the individual stimulations against the unstimulated control; $p<0.05$ was considered statistically significant. Although statistically analysis of the co-immunoprecipitations were performed the data is not presented in this thesis. Due to the limitations of the immunoprecipitation techniques any analysis can only be semi-quantitative and therefore the presentation of statistical data relating to these could be misleading.

5.4 Expression of Accessory Proteins in Myometrial Cell Cultures.

Prior to determining if the KCNMA1 and CACNA1C exist within a macromolecular complex and what form this complex takes it was first necessary to determine if the myometrial cells expressed the putative accessory proteins, $G_{\alpha s}$, Cav-1 and β_2AR and if either TNF or TSA had an effect of the level of expression of these proteins. To this end Western blots were carried out for each of the accessory proteins.

5.4.1 Expression of $G_{\alpha s}$ in Myometrial Cell Cultures

After whole cell lysis protein extraction, $G_{\alpha s}$ was detected in all the samples (Figure 5.3A left panel). On the western blots two isoforms of the $G_{\alpha s}$ protein were detected, a ~54kDa and a ~46kDa variant (Figure 5.3A left panel), these are designated the long and short isoforms of $G_{\alpha s}$ respectively (Northup, Sternweis *et al.* 1980, Mumby, Kahn *et al.* 1986, Olney, Tsuchiya *et al.* 1996, Pollard, Krainer *et al.* 2002). These two isoforms of $G_{\alpha s}$, have been shown to be generated by alternative splicing with the short form being generated by the exclusion of exon 3 (Kozasa, Itoh *et al.* 1988). These two different isoforms have been shown to have different regulatory functions, with the longer form more able to support hormone-stimulated AC activity (Sternweis, Northup *et al.* 1981), and preferentially coupling to β-adrenergic receptors (Yagami 1995) in comparison to the short form. Expression of these two isoforms has been found to be tissue specific (Novotny and Svoboda 1998). Within the myometrium it has been reported that both the long and short $G_{\alpha s}$ isoforms are expressed at a considerably higher level in pregnant myometrium versus non-pregnant and in labouring myometrium the levels are reduced to that seen in non-pregnant (Europe-Finner, Phaneuf *et al.* 1994, Europe-Finner, Phaneuf *et al.* 1996).

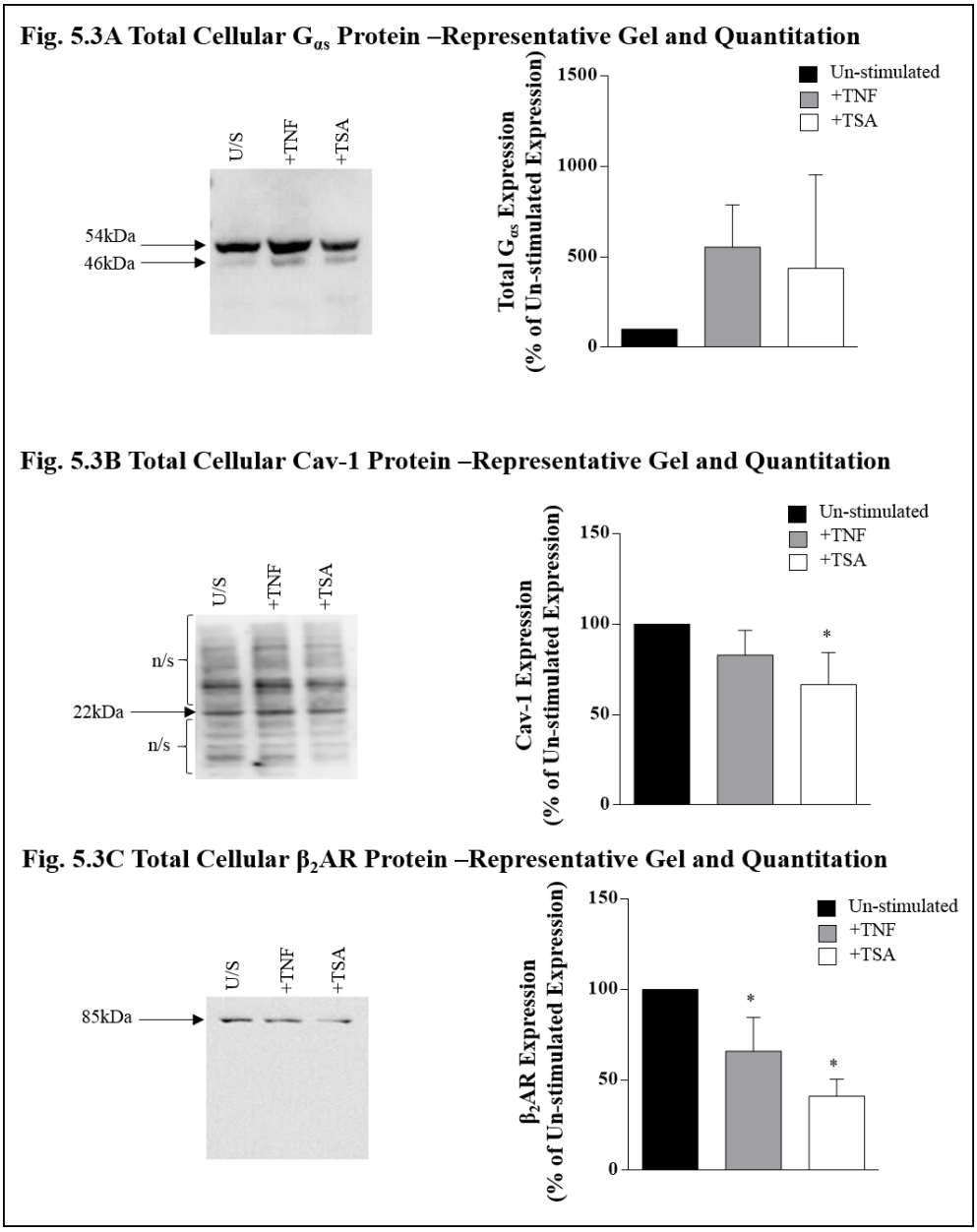


Figure 5.3 Expression of $G_{\alpha s}$, Cav-1 and β_2AR in Myometrial Cell Cultures
Myometrial cell cultures were stimulated with 10ng/ml TNF for 1 hour, 100ng/ml TSA for twenty-four hours, or left un-stimulated. Protein was extracted, separated by SDS PAGE and western blotted. Manual quantification of the relative band intensities were used to quantify the level of expression and this was expressed as a percentage of the un-stimulated expression (n = 3) (A) Left Panel Representative $G_{\alpha s}$ blot, both the long (54kDa) and short (46kDa) $G_{\alpha s}$ isoforms were detected, Right panel – Quantification. There was no change in total $G_{\alpha s}$ expression with either treatment. (B) Left Panel Representative Cav-1 blot, Right panel – Quantification. TSA caused a significant down regulation of Cav-1 (*$p<0.05$). (C) Left Panel Representative β2AR blot, Right panel – Quantification. Both TNF and TSA caused a significant down regulation of β2AR (*$p<0.05$)

There was no significant change in the total level of $G_{\alpha s}$ protein detected after either TNF or TSA stimulation compared to the un-stimulated controls (Figure 5.3A right panel).

5.4.2 Expression of Caveolin 1 (Cav-1) in Myometrial Cell Cultures

As mentioned previously caveolae are small flask-shaped invaginations of the cell membrane, the formation of these structures requires the expression of caveolin proteins (Fra, Williamson *et al.* 1995, Drab, Verkade *et al.* 2001, Razani, Engelman *et al.* 2001). Caveolins are a family of three integral membrane proteins, Cav-1, Cav-2 and Cav-3, which all form hairpin loops which are inserted into the membrane with the N and C termini both facing the cytoplasmic side of the membrane (Monier, Parton *et al.* 1995). These caveolin proteins form oligomers and when these associate with cholesterol and sphingolipids they result in the formation of caveolae. In smooth muscle Cav-1 has been shown to be crucial for the formation of caveolae.

After whole cell lysis protein extraction, Cav-1 was detected in all the samples (Figure 5.3B left panel). There was no significant change in the level of Cav-1 protein detected after TNF stimulation compared to the un-stimulated controls (Figure 5.3B right panel). After TSA stimulation, however, the amount of Cav-1 protein detected was significantly reduced ($p<0.05$; Figure 5.3B right panel).

A large number of bands were observed on the Cav-1 western blots, these are labelled non-specific (n/s; Figure 5.3B left panel). However, there is evidence to suggest that Cav-1 can form stable hetero-oligomeric complexes with Cav-2 (Scherer, Lewis *et al.* 1997) and this may explain the banding pattern seen on the western blot. The proteins represented by these other bands have not been conclusively identified for this thesis.

5.4.3 Expression of β_2-Adrenergic Receptor in Myometrial Cell Cultures

The β_2AR is a G-protein coupled receptor located within the cell membrane which binds Norepinephrine and Epinephrine. When stimulated, the β_2AR results in the hyperpolarization of smooth muscle, an effect mediated by the augmentation of potassium ion conductance (Kotlikoff and Kamm 1996). Briefly, stimulation of the β_2AR activates AC via $G_{\alpha s}$ which in turn converts ATP to cAMP. cAMP then catalyses the activation of PKA which in turn phosphorylates key proteins, including the KCNMA1 channel. Phosphorylation of the KCNMA1 channel leads to its activation and hence relaxation of the muscle (Kume, Takai *et al.* 1989, Tanaka, Yamashita *et al.* 2003). β_2AR agonists such

as ritrodine were used to inhibit labour by causing relaxation in the smooth muscle of the uterus (Haram, Mortensen *et al.* 2003).

Using whole cell protein extracts, β_2AR was detected in all the samples (Figure 5.3C left panel). There was a significant decrease in the level of β_2AR protein detected after both TNF and TSA stimulation compared to the un-stimulated controls ($p<0.05$; Figure 5.3C right panel). This reduction in expression was greatest in the TSA stimulated samples.

5.5 Optimisation of Immunoprecipitation

5.5.1 Optimisation of Antibody Binding

Each step of the antibody binding and crosslinking process was tested using two amounts of both the KCNMA1 and CACNA1C N-terminal antibodies (2.5µg and 5.0µg). The effects of adding either reducing or non-reducing sample buffer to the sample prior to analysis by SDS PAGE were also compared. The first step in this process was to verify that the antibody was successfully bound to the beads. After crosslinking the antibody to the beads, the supernatant, termed the flow through, was collected. This would contain any antibody that has not bound to the beads. Next, non-cross-linked antibody was eluted from the beads using elution buffer (0.1% (v/v) HCl pH2.0), this step was repeated and the two elutions collected. Finally a sample of the beads was collected, this should contain cross-linked antibody.

Following co-immunoprecipitaion the presence of the immunoprecipitating antibody in the protein eluate can mask specific signals from the subsequent western blot. This is due to the fact that the HRP-conjugated secondary antibody can bind to the immunoprecipitation antibody as well as the primary antibody utilised in the western blot. Depending on the size of the protein that is being detected the immunopreciptating antibody IgG heavy and light chains can mask the specific protein signal. The apparent size of these IgG bands depends on if the antibody is intact or reduced. If the antibody is intact, both the heavy and light chains will migrate together through the gel and will be detected higher on the membrane (~75kDa) than reduced antibody where the heavy and light chains migrate separately (50-75kDa and ~25-kDa respectively). To determine the level of interference from both reduced and intact contaminating immunoprecipitating

antibody, each sample was divided into two aliquots. One aliquot was diluted in non-reducing laoding buffer and the second in reducing loading buffer. The SDS and DTT in the reducing buffer combined with boiling the sample breaks the secondary and tertiary structure of the proteins resulting in the separate migration of the heavy and light IgG chains, whilst non-reducing loading buffer will leave the antibody intact.

Each of the samples was analysed by SDS PAGE on an 8% resolving gel, transferred to nitrocellulose membrane and then after blocking, incubated with the appropriate secondary antibody to detect the presence of any primary antibody in the sample.

There was no antibody detected in the flow through samples, indicating that the antibody had successfully bound to the beads. The first elution contained a detectable amount of antibody indicating that the crosslinking was not 100% efficient. The second elution contained much less antibody demonstrating that the elutions have removed the majority of non-crosslinked antibody. Finally the beads did contain detectable antibody demonstrating that a portion of the antibody had been successfully cross-linked to the beads (Figure 5.4A).

Two different amounts of antibody were examined 2.5µg or 5µg per immunoprecipitation to determine if this affected the efficiency of the crosslinking. There were no differences in the amount of antibody detected at each of the stages between these two groups (Figure 5.4A).

Intact antibody was detect high up on the membrane and therefore, will not interfere with the visualisation of the specific proteins of interest. Reduced antibody, however, was detected in the same region as the specific proteins are expected to be and so these will interfere with the interpretation of the co-immunoprecipitation blots (Figure 5.4A).

Fig. 5.4A Verification of Antibody Cross-linking

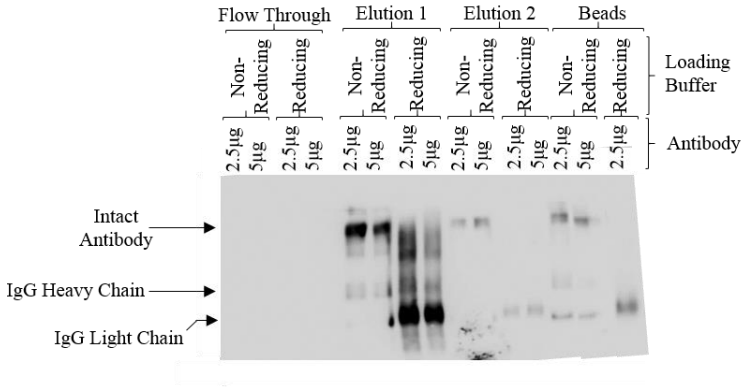


Fig. 5.4B Verification of Protein Immunoprecipitation

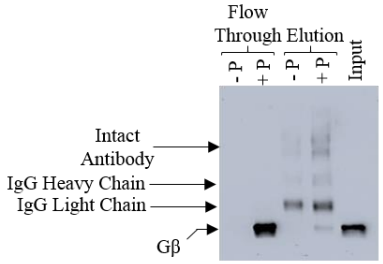


Fig. 5.4C Protein A HRP Trial

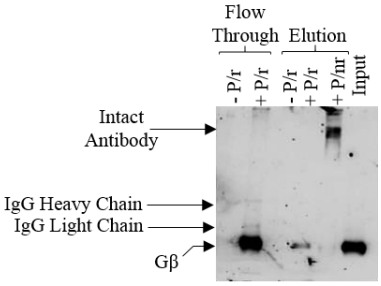


Figure 5.4 Optimisation of Co-Immunoprecipitation Protocol

After crosslinking of the Gβ antibody to the beads the crosslinked beads and antibody were incubated both with and without protein (+p and –p respectively), un-bound protein was removed and bound protein was eluted from the beads. (A) Verification of Antibody Cross-linking. No antibody was detected in the wash (flow through) following antibody crosslinking. Antibody was detected following elution from the beads (Eluion1; Elution2). Antibody was detected in the bead sample indicating the crosslinking was partially effective. Detected antibody was high on the blot in samples diluted in non-reducing loading buffer and the heavy and light IgG chains were detected separately in samples diluted in reducing loading buffer. (B) Verification of Protein Immunoprecipitation. No antibody is detected in either of the flow though lanes indicating that the antibody is crosslinked to the beads. Gβ is detected in the +p flow through lane. In the -p elution antibody is detected indicating that the elution removes some of the crosslinked antibody, this was also seen in the +p elution. In the +p elution Gβ was also detected indicating that the immunoprecipitation was successful. (C) Protein-A HRP Trial. Flow Through and Elution (+p; –p) samples were diluted in reducing loading buffer, one elution sample (+p/nr) was diluted in non-reducing sample buffer. No antibody was detected in either of the flow though lanes indicating that the antibody is crosslinked to the beads. Gβ is detected in the +p flow through lane. No antibody was detected in the –p and +p elutions, Gβ was detected in the +p elution. In the +p/nr elution antibody is detected indicating that antibody is present but just not detected by protein-A HRP. +p – +0.3mg protein; -p – no protein

5.5.2 Optimisation of Immunoprecipitation Western Blots

To ensure the antibodies did not obscure data interpretation on the blot, they were chemically cross-linked to the beads. To determine if this cross-linking had been effective, a test immunoprecipitation was carried out using Gβ antibody.

The immunoprecipitation was carried out both with 500µl IP Lysis / Wash buffer (without protein: -p) and with 0.3mg total cellular protein in 500µl IP Lysis / Wash buffer (with protein: +p). Under both these conditions samples were collected at each step of the immunoprecipitation protocol namely, the flow through from the beads following the first incubation and the elution of the immunoprecipitated protein from the beads. Reducing loading buffer was added to all the samples which were subsequently analysed by SDS PAGE followed by western blotting for Gβ.

In the "without protein" (-p) sample there were no immunoprecipitating antibody IgG bands detected on the blot in the flow through lanes, however, in the elution sample lanes immunoprecipitating antibody heavy and light chain IgG bands were detected on the blot (Figure 5.4B). The lack of bands in the flow through sample indicates that the antibody cross-linking experiment was effective in removing contaminating immunoprecipitating antibody. The IgG bands in the elution samples, however, indicate that the elution procedure removes some of the cross-linked antibody from the beads.

In the "with protein" (+p) sample there was no immunoprecipitating antibody IgG bands detected on the blot in the flow through lanes, however, in this sample a band for Gβ was detected. This may indicate that too much protein has been added to the immunoprecipitation. In the elution lane both immunoprecipitating antibody IgG bands and a Gβ band were detected on the blot (Figure 5.4B). This indicates that the immunoprecipitation is working and recovering the expected protein.

As a final optimisation step, a different secondary antibody was trialled. The traditional secondary antibodies used for western blotting recognise both intact and reduced antibody which can lead to high background especially on western blots from immunoprecipitated protein. As has been explained previously this high background can mask the signals of interest on the western blot. Lal *et al.* demonstrated that this issue can be resolved by using Protein-A HRP as an alternative secondary. Protein-A HRP recognises almost

exclusively intact antibody. As the SDS and DTT in the reducing buffer combined with boiling the sample breaks the secondary and tertiary structure of the proteins this renders the immunoprecipitating antibody undetectable by the Protein-A HRP secondary (Lal, Haynes *et al.* 2005).

To determine if a Protein-A HRP conjugated secondary antibody could overcome the issues associated with immunoprecipitating antibody contamination of the immunoprecipitated protein the follow experiment was carried out. Immunoprecipitations were performed with both with 500µl IP Lysis / Wash buffer (without protein: -p) and with 0.3mg total cellular protein in 500µl IP Lysis / Wash buffer (with protein: +p). Under both these conditions samples were collected at each step of the immunoprecipitation protocol namely, the flow through from the beads following the first incubation and the elution of the immunoprecipitated protein from the beads. The 'with protein' (+p) elution sample was then divided equally into two aliquots. One aliquot was diluted in reducing sample buffer (+p/r) which should render the immunoprecipitating antibody undetectable by Protein-A HRP. The second aliquot was diluted in non-reducing sample buffer (+p/nr) meaning that the immunoprecipitating antibody is detectable by Protein-A HRP. The remaining samples were diluted in reducing sample buffer (-p/r; +p/r). All the resulting samples were then analysed by SDS PAGE followed by western blotting using the Protein-A HRP secondary antibody.

In all the "with protein" (+p) samples, Gβ protein was detected both in the flow through and in the elution (Figure 5.4C), this indicates that the protein-A HRP secondary is of a comparable sensitivity to the traditional HRP-conjugated secondary's routinely used for western blotting.

In the sample diluted in non-reducing loading buffer contaminating antibody was detected high up on the membrane (Figure 5.4C). However, in all the samples diluted in reducing loading buffer there was no contaminating IgG detected (Figure 5.4C). This demonstrates that if reducing loading buffer is used in combination with the Protein-A HRP secondary the high background due to the immunoprecipitating antibody is eliminated.

5.6 Association of KCNMA1 and CACNA1C with Other Proteins in Myometrial Cell Cultures.

KCNMA1 and CACNA1C present within myometrial cells, were extracted from cell cultures which were either left unstimulated, or stimulated with 10ng/ml TNF or, 2µg/ml TSA. The protein was extracted using a non-denaturing IP Lysis buffer (pH7.4, 25mM Tris, 150mM NaCl, 1mM EDTA, 1% (v/v) NP40, 5% (v/v) glycerol; Pierce IP/Co-IP Kit). The protein was quantified using a nanophotometer, by UV absorption at A280nm. Once the protein was extracted the Pierce IP/Co-IP kit was used to immunoprecipitate protein complexes containing KCNMA1 or CACNA1C protein by utilising KCNMA1 and CACNA1C specific antibodies. Non-specific IgG was also used to immunoprecipitate the protein as a measure of the non-specific background signal from the immunoprecipitations. Finally proteins which had not been immunoprecipitated was used as an input control to demonstrate that the protein of interest was present in the sample. Pre-cast 4-20% SDS PAGE gels were loaded with input protein, IgG, KCNMA1 and CACNA1C immunoprecipitated protein and then probed with each of the putative accessory proteins. The exceptions to this were the KCNMA1 N-terminal IP subsequently western blotted with the KCNMA1 N-terminal antibody and the IP subsequently blotted with $G_{\alpha s}$, these were incorrectly loaded as follows; input, KCNMA1, IgG, CACNA1C due to limited samples it was not possible to re-run these blots.

5.7 Immunoprecipitation Control Blots

As controls, western blots were also probed with the same antibody that was used to immunoprecipitate the protein.

When proteins immunoprecipitated with the KCNMA1 N-terminal antibody was western blotted using the same KCNMA1 N-terminal antibody there were bands detected at 138kDa, 65kDa and 50kDa as well as some non-specific (n/s) bands (Figure 5.5A). The 138kDa, 65kDa and 50kDa bands correlate with bands detected by the KCNMA1 N-terminal antibody in Chapter 4 Figure 4.4. The controls and tests of the immunoprecipitation, described previously, suggest that the interactions are specific for the KCNMA1 protein. Therefore, these bands are representative of full length KCNMA1 channel (138kDa; Figure 5.5A), and two C-terminally truncated KCNMA1 channel (50kDa and 65kDa; Figure 5.5A). Also there is some KCNMA1 banding in the IgG lanes

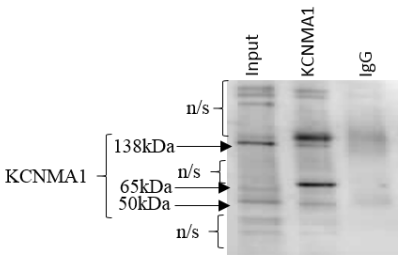
(Figure 5.5A) which would indicate that there has been some non-specific binding by the IgG antibody during the immunoprecipitation step, which has not be removed by the subsequent washing steps.

There was banding for the KCNMA1 protein in all three of the immunoprecipitations carried out using the KCNMA1 N terminal antibody (un-stimulated, +TNF and +TSA) indicating that all the immunoprecipitations have been successful (Figure 5.5A).

When the protein immunoprecipitated with the CACNA1C N-terminal antibody was western blotted using the same CACNA1C N-terminal antibody there were bands detected at 240kDa and 190kDa as well as some non-specific (n/s) bands in the immunoprecipitation lanes (Figure 5.5B). The 240kDa and 190kDa bands correlate with bands detected by the CACNA1C N-terminal antibody detailed in Chapter 4 Figure 4.6. The controls and tests of the immunoprecipitation run previously would again suggest that the interactions are specific for the CACNA1C protein. Therefore, these bands are representative of full length CACNA1C channel (240kDa; Figure 5.5B), and a C-terminally truncated CACNA1C channel (190kDa; Figure 5.5B). There are also some intense non-specific bands detected, these are below the CACNA1C bands and are not of the correct size to be the IgG heavy or light chains. It is possible that these bands represent IgG heavy chain dimers, however, it has been demonstrated that the protein-A HRP secondary used does not detect the reduced IgG heavy and light chains. Therefore, at present the identity of these bands is unclear. Some CACNA1C banding was also noted in the IgG lanes (Figure 5.5A) indicating that there has been some non-specific binding during the immunoprecipitation step, which was not removed during the washing steps.

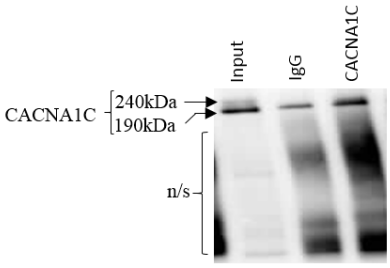
There was banding for the CACNA1C in each of the immunoprecipitations carried out using the CACNA1C N-terminal antibody (un-stimulated, +TNF and +TSA) indicating that the immunoprecipitations have been successful (Figure 5.5B).

Fig. 5.5A KCNMA1 Control Immunoprecipitation – Representative Blots



IP – KCNMA1 N-terminal
WB – KCNMA1 N-Terminal

Fig. 5.5B CACNA1C Control Immunoprecipitation – Representative Blots



IP – CACNA1C N-terminal
WB – CACNA1C N-Terminal

Figure 5.5 Immunoprecipitation Controls
Myometrial cell cultures were stimulated with 10ng/ml TNF, 100ng/ml TSA, or left un-stimulated. Protein was extracted, immunoprecipitated with KCNMA1 N-terminal, CACNA1C N-terminal and IgG antibodies then separated by SDS PAGE and western blotted. (A) Representative blots of KCNMA1 N-terminal and IgG immunoprecipitations western blotted with KCNMA1 N-terminal antibody. KCNMA1 protein was detected in all KCNMA1 N-terminal immunoprecipitations indicating that the immunoprecipitation was successful. (B) Representative blots of CACNA1C N-terminal and IgG immunoprecipitations western blotted with CACNA1C N-terminal antibody. CACNA1C protein was detected in all CACNA1C N-terminal immunoprecipitations indicating that the immunoprecipitation was successful. IP – immunoprecipitation, WB – western blot, n/s – non-specific.

5.7.1 The KCNMA1 Channel is Found Associated with $G_{\alpha s}$ in Myometrial Cell Cultures

When the protein immunoprecipitated with the KCNMA1 N-terminal antibody was western blotted using the $G_{\alpha s}$ antibody there was a band detected at 54kDa as well as some non-specific (n/s) bands (Figure 5.6A). This 54kDa band correlates with the band detected by the $G_{\alpha s}$ antibody in Figure 5.3A. The presence of this $G_{\alpha s}$ specific band indicates that $G_{\alpha s}$ is associated with the KCNMA1 channel in un-stimulated samples. Following exposure to TNF and TSA the KCNMA1 channel was again found associated with $G_{\alpha s}$. Within the TNF-stimulated samples there was also a second $G_{\alpha s}$ band detected at 46kDa, this correlates with the band detected by the $G_{\alpha s}$ antibody in Figure 5.3A. Although the association was evident in each of the repeats the intensity of the band

observed varied between repeats. There was no corresponding bands detected in the IgG IP lanes indicating a low level of background signal (Figure 5.6A).

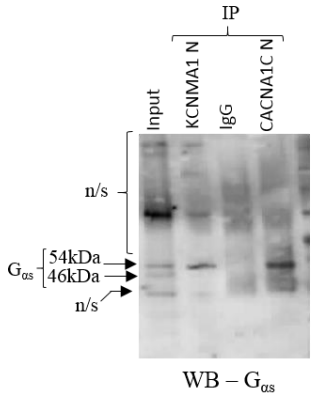
5.7.2 The CACNA1C Channel is not Found Associated with $G_{\alpha s}$ in Myometrial Cell Cultures

When the protein immunoprecipitated with the CACNA1C N-terminal antibody was western blotted using the $G_{\alpha s}$ antibody there was no banding observed at either 54 or 46kDa, there was some non-specific banding detected (Figure 5.6A). Indicating that $G_{\alpha s}$ is not found associated with CACNA1C in un-stimulated samples. Following exposure to TNF there was no association between the CACNA1C and $G_{\alpha s}$ evident. Exposure to TSA, however, resulted in the association of $G_{\alpha s}$ and the CACNA1C as evidenced by the observation of a 54kDa band (Figure 5.6A). Unlike the KCNMA1 channel there was no association found between the CACNA1C and the short $G_{\alpha s}$ isoform. The results from these co-immunoprecipitations were inconsistent, and the association between $G_{\alpha s}$ and the CACNA1C was only evident in one repeat from the TSA stimulated samples. The input band was clear on these blots indicating that the lack of $G_{\alpha s}$ band in the CACNA1C IP lanes was not a consequence of an unsuccessful western blot but rather that $G_{\alpha s}$ is not routinely associated with the CACNA1C in these primary myocytes. There was no corresponding bands detected in the IgG IP lanes indicating a low level of background signal (Figure 5.6A).

5.7.3 Cav-1 Immunoprecipitations

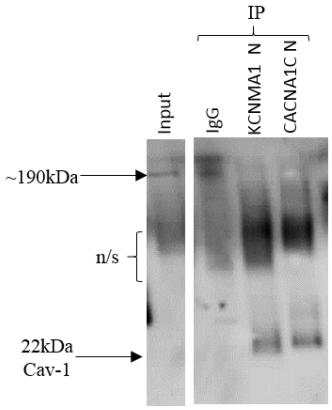
It is pertinent to note that in the input lanes on these blots the Cav-1 band is high on the blots this, may be due to a failure to dissociate the stable hetero-oligomeric complexes which Cav-1 has been show to form (Scherer, Lewis *et al.* 1997). However, this high molecular weight band has not been positively identified for this thesis. In the input lanes there was no band detected corresponding to the 22kDa Cav-1 protein, however following IP with either the KCNMA1 channel or CACNA1C antibodies and subsequent western blotting with the Cav-1 antibody, bands were detected at 22kDa, which corresponds to the accepted size of the Cav-1 protein. The following discussions of the association of the KCNMA1 and CACNA1C with Cav-1 are based on the presence of the 22kDa bands.

Fig. 5.6A $G_{\alpha s}$ Co-Immunoprecipitation – Representative Blot



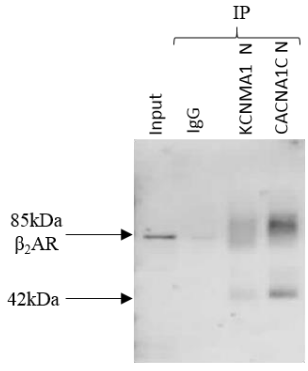
WB – $G_{\alpha s}$

Fig. 5.6 B Cav-1 Co-Immunoprecipitation – Representative Blot



WB – Cav-1

Fig. 5.6C β_2AR Co-Immunoprecipitation – Representative Blot



WB – β_2AR

Figure 5.6 KCNMA1 N-terminal and CACNA1C N-terminal Associations with $G_{\alpha s}$, Cav-1 and β_2AR
Myometrial cell cultures were stimulated with 10ng/ml TNF, 100ng/ml TSA, or left un-stimulated. Protein was extracted, immunoprecipitated with KCNMA1 N-terminal, CACNA1C N-terminal and IgG antibodies then separated by SDS PAGE and western blotted (n = 3). (A) Representative blots of KCNMA1 N-terminal, CACNA1C N-terminal and IgG immunoprecipitations western blotted with $G_{\alpha s}$ antibody (B) Representative blots of KCNMA1 N-terminal, CACNA1C N-terminal and IgG immunoprecipitations western blotted with Cav-1 antibody. Cav-1 protein was detected in all KCNMA1 N-terminal and CACNA1C N-terminal immunoprecipitations. (C) Representative blots of KCNMA1 N-terminal, CACNA1C N-terminal and IgG immunoprecipitations western blotted with β_2AR antibody. B_2AR protein was detected in all KCNMA1 N-terminal and CACNA1C N-terminal immunoprecipitations. IP – immunoprecipitation, WB – western blot, n/s – non-specific.

5.7.4 The KCNMA1 Channel is Found Associated with Cav-1 in Myometrial Cell Cultures

When the protein immunoprecipitated with the KCNMA1 N-terminal antibody was western blotted using the Cav-1 antibody there was a band detected at 22kDa as well as some non-specific (n/s) bands (Figure 5.6B). This 22kDa band correlates with the band detected by the Cav-1 antibody in Figure 5.3B. The presence of this Cav-1 specific band indicates that Cav-1 is associated with the KCNMA1 channel in un-stimulated samples. Following exposure to TNF and TSA, the KCNMA1 channel was again found associated with Cav-1. There was no corresponding bands detected in the IgG IP lanes indicating a low level of background signal (Figure 5.6A).

5.7.5 The CACNA1C Channel is Found Associated with Cav-1 in Myometrial Cell Cultures

When the protein immunoprecipitated with the CACNA1C N-terminal antibody was western blotted using the Cav-1 antibody there was a band detected at 22kDa as well as some non- specific (n/s) bands (Figure 5.6B). This 22kDa band correlates with the band detected by the Cav-1 antibody in Figure 5.3B. The presence of this Cav-1 specific band indicates that Cav-1 is associated with the CACNA1C channel in un-stimulated samples. Following exposure to TNF and TSA, the CACNA1C channel was again found associated with Cav-1.

There was no corresponding bands detected in the IgG IP lanes indicating a low level of background signal (Figure 5.6A).

5.7.6 The KCNMA1 Channel is Found Associated with β_2-Adrenergic Receptor in Myometrial Cell Cultures

When the protein immunoprecipitated with the KCNMA1 N-terminal antibody was western blotted using the β_2AR antibody there was a band detected at 85kDa (Figure 5.6C). This 85kDa band correlates with the band detected by the β_2AR antibody in Figure 5.3C. The presence of this β_2AR specific band indicates that β_2AR is associated with the KCNMA1 channel in un-stimulated samples. Following exposure to TNF and TSA, the KCNMA1 channel was again found associated with β_2AR. There was no corresponding

bands detected in the IgG IP lanes indicating a low level of background signal (Figure 5.6A).

5.7.7 The CACNA1C Channel is Found Associated with β_2-Adrenergic Receptor in Myometrial Cell Cultures

When the protein immunoprecipitated with the CACNA1C N-terminal antibody was western blotted using the β_2AR antibody there was a band detected at 85kDa (Figure 5.6C). This 85kDa band correlates with the band detected by the β_2AR antibody in Figure 5.3C. The presence of this β_2AR specific band indicates that β_2AR is associated with the CACNA1C channel in un-stimulated samples. A second band at ~42kDa was also observed within the CACNA1C IP lanes. It is possible that the 85kDa band represents a β_2AR dimer whilst this smaller band represents a β_2AR monomer (Angers, Salahpour *et al.* 2000). Salahpour *et al.* demonstrated that homodimerisation of the β_2AR was a prerequisite for cell surface targeting. Therefore the 42kDa putative monomer band may indicate that this β_2AR is internalised, the implications of this will be explored in the discussion. Following exposure to TNF and TSA, the CACNA1C channel was again found associated with β_2AR and again both the 85kDa and 42kDa were present. There was no corresponding bands detected in the IgG IP lanes indicating a low level of background signal (Figure 5.6C).

5.7.8 The KCNMA1 N- and C-termini are Associated in Myometrial Cell Cultures

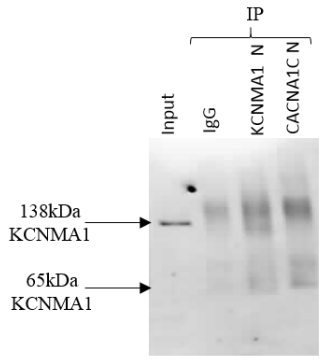
When the protein immunoprecipitated with the KCNMA1 N-terminal antibody was western blotted using the KCNMA1 C-terminal antibody the 138kDa band detected (Figure 5.7A) was very faint, and no band was detected at 65kDa. This 138kDa band correlates with the band detected by the KCNMA1 C-terminal antibody in Chapter 4 Figure 4.5. Neither TNF nor TSA stimulation had any effect on this association.

5.7.9 The CACNA1C N-terminus and the KCNMA1 C-terminus are Associated in Myometrial Cell Cultures

When the protein immunoprecipitated with the CACNA1C N-terminal antibody was western blotted using the KCNMA1 C-terminal antibody there was a 138kDa band and a 65kDa band detected in the un-stimulated samples (Figure 5.7A). Both the 138kDa and

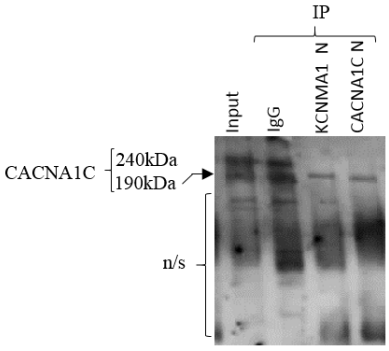
65kDa bands correlate with the band detected by the KCNMA1 C-terminal antibody in Chapter 4 Figure 4.5. The presence of this KCNMA1 specific band indicates that the KCNMA1 C-terminal is associated with the CACNA1C N-terminal in un-stimulated samples. The presence of the 65kDa band indicates there is an association between the CACNA1C and KCNMA1 channels which lack the N-terminal region including the pore. The significance of this association between the CACNA1C and non ion conducting KCNMA1 channel forms is still to be elucidated. Neither TNF nor TSA stimulation had any effect on this association.

Fig. 5.7A KCNMA1 C-terminal Co-Immunoprecipitation – Representative Blot



WB – KCNMA1 C-terminal

Fig. 5.7B CACNA1C C-terminal Co-Immunoprecipitation – Representative Blot



WB – CACNA1C C-terminal

Figure 5.7 KCNMA1 N-termial and CACNA1C N-terminal Associations with the KCNMA1 C-terminal and CACNA1C C-terminal

Myometrial cell cultures were stimulated with 10ng/ml TNF, 100ng/ml TSA, or left un-stimulated. Protein was extracted, immunoprecipitated with KCNMA1 N-terminal, CACNA1C N-terminal and IgG antibodies then separated by SDS PAGE and western blotted. (A) Representative blots of KCNMA1 N-terminal, CACNA1C N-terminal and IgG immunoprecipitations western blotted with KCNMA1 C-terminal antibody. (B) Representative blots of KCNMA1 N-terminal, CACNA1C N-terminal and IgG immunoprecipitations western blotted with CACNA1C C-terminal antibody. IP – immunoprecipitation, WB – western blot, n/s – non-specific.

5.7.10 The CACNA1C N-terminus and the CACNA1C C-terminus are Associated in Myometrial Cell Cultures

When the protein immunoprecipitated with the CACNA1C N-terminal antibody was western blotted using the CACNA1C C-terminal antibody there was a band detected in the un-stimulated samples (Figure 5.7B). This band appears to lie between the 240kDa and 190kDa bands detected in the input lane. As explained above this may either be an artefact of the gel or it may represent a CACNA1C which has lost a smaller portion of the C-terminal region during the extraction of immunoprecipitation protocols. The presence of this CACNA1C specific band indicates that the CACNA1C N-terminal is associated with the CACNA1C C-terminal in un-stimulated samples. Neither TNF nor TSA stimulation had any effect on this association. Also there is some CACNA1C banding in the IgG lanes (Figure 5.5A) which would indicate that there has been some non-specific binding during the immunoprecipitation step, which has not be removed by the subsequent washing steps.

5.8 Discussion

5.8.1 $G_{\alpha s}$ Expression in Myometrial Cell Cultures.

The results reported herein appear to conflict with previous research which has reported repression of $G_{\alpha s}$ expression both with TNF stimulation and in labouring myometrial biopsies, however this previous research either examined the effect of TNF on $G_{\alpha s}$ promoter activity (Chapman, Smyrnias *et al.* 2005, Webster, Waite *et al.* 2013) or compared the expression of $G_{\alpha s}$ in non-pregnant, pregnant non-labouring and pregnant labouring biopsy samples. As such neither can be directly correlated to the data presented here.

There is a paucity of information reported regarding the effect of TSA on the expression of $G_{\alpha s}$ although Europe-Finner *et al.* reported an up-regulation of $G_{\alpha s}$ in pregnant myometrium and Webster *et al.*reported that $G_{\alpha s}$-luciferase reporter plasmid promoter activity is induced by TSA which would suggest that acetylation of the promoter and/ or other factors regulating this region are needed for expression (Europe-Finner, Phaneuf *et al.* 1993, Webster, Waite *et al.* 2013). The data reported above, again appears to conflict with these previous studies. However, Webster *et al.* looked at the effect of TSA on the

$G_{\alpha s}$ promoter and induction of a promoter does not necessarily lead straight to an increase in protein. Increasing levels of protein are dependent on increased translation and possibly increased retention or decreased removal of the protein as well as an increase in the transcription of the mRNA. These other factors may explain the differences seen between the effect of TSA on $G_{\alpha s}$ protein levels and its effect on the promoter.

5.8.2 Cav-1 Expression in Myometrial Cell Cultures.

TNF has been reported to both repress the expression of Cav-1 in HUVEC cells (Sun, Muller *et al.* 2003) and to have no effect on Cav-1 expression in Bovine aortic endothelial cells (Wang, Wang *et al.* 2008) therefore the effect of TNF on Cav-1 appears to be tissue specific. Cav-1 has been reported to gradually increase during pregnancy up to the start of labour and then decrease to levels seen in non-pregnant myometrium (Turi, Kiss *et al.* 2001). The data reported herein demonstrate that TNF has no significant effect on the expression of Cav-1. In contrast to this observation TSA appears to cause a small but statistically significant reduction in expression. This would suggest that within myometrial smooth muscle cells TNF is not the driving force for the reduction in Cav-1 seen at parturition.

Boopathi *et al.* demonstrated that the transcritption factor GATA-6 is responsible for the transcriptional repression of Cav-1 (Boopathi, Gomes *et al.* 2011) and Caslini *et al.* demonstrated that expression of GATA-6 was restored after TSA treatment (Caslini, Capo-chichi *et al.* 2006). This was shown to be via the increased acetylation of H3 (lys9 and lys4), H4 (lys5, 8, 12 and 16) and H3 Lysine K4 methylation which led to a more open gene structure and hence increased the transcription of GATA-6. The effect of TSA could therefore be via its promotion of the up-regulation of GATA-6 and then the subsequent repression of Cav-1 expression by GATA-6. The data reported herein is in broad agreement with these previous findings.

5.8.3 β_2-Adrenergic Receptor Expression in Myometrial Cell Cultures.

As β_2AR agonists have been utilised as tocolytics it would be expected that TNF would cause a down regulation in the expression of the β_2AR and that is indeed the case in data described above.

As the β_2AR is well documented to support relaxation (Kotlikoff and Kamm 1996, Liu, Nwosu *et al.* 1998, Chanrachakul, Matharoo-Ball *et al.* 2003, Tanaka, Yamashita *et al.* 2003) and TSA has also been shown to cause relaxation (Moynihan, Hehir *et al.* 2008, Webster, Waite *et al.* 2013) it appears contradictory, therefore, that TSA would down-regulate the β_2AR. In fact there has been a study which indicates that pan-acetylation of key histones, namely Histone 3 and Histone 4, mediates an increase in β_2AR transcription although this research was conducted in murine T_H1 and T_H2 cells and therefore this may be a tissue-specific effect. The findings reported above, however, indicate that in the case of cultured myometrial smooth muscle cells TSA causes a significant down regulation of the β_2AR. Although outside the scope of this thesis, the mechanism by which TSA supresses' β_2AR expression is certainly worthy of further investigation.

5.9 Protein-Protein Interactions

5.9.1 $G_{\alpha s}$ Interactions

$G_{\alpha s}$ is a heterotrimeric G protein subunit that activates AC and hence the cAMP-dependant pathway, it is activated by G protein-coupled receptors. Upon activation, proteins such as $G_{\alpha s}$, which are involved in signal transduction, can undergo regulated and reversible translocation within the cell, moving between the membrane, cytoplasm or nucleus. Upon activation, $G_{\alpha s}$ has been shown to reversibly detach from the membrane and become a soluble protein (Ransnäs, Svoboda *et al.* 1989, Levis and Bourne 1992, Wedegaertner, Bourne *et al.* 1996). Some G proteins and G protein coupled receptors are concentrated within caveolae (Chang, Ying *et al.* 1994, Chun, Liyanage *et al.* 1994, Lisanti, Scherer *et al.* 1994, Chakrabarti, Chang *et al.* 2010). The nature of $G_{\alpha s}$ protein interactions is therefore transient and non-covalent in nature which explains the difficulty in getting consistent co-immunoprecipitation repeats for this protein.

$G_{\alpha s}$ has been shown to stimulate dihydropyridine-sensitive calcium channels, such as the CACNA1C (Yatani, Imoto *et al.* 1988). In these skeletal muscle T tubules, however, the CACNA1C displayed notably different kinetics and ion conductance. By using patch clamp experiments that study demonstrated addition of exogenous purified $G_{\alpha s}$ protein increased the open probability of these channels. They concluded that this activation of the CACNA1C by $G_{\alpha s}$ was via a second pathway which is distinct from the activation of

the CACNA1C by PKA as there was no ATP in the system. However, this study was performed on T-tubule calcium channels which had been reconstituted into lipid bilayers and so have been removed from their cellular context. This may explain the difference in the kinetics and ion conductance observed and may affect the effect of $G_{\alpha s}$ on the channel.

G proteins have also been shown to have a role in both the stimulatory and inhibitory regulation of calcium activated potassium channels such as the KCNMA1 channel (Kume, Graziano *et al.* 1992). This regulation of calcium-activated potassium channels was again two-fold as described for the CACNA1C. In one pathway after activation of the G-protein coupled receptor, $G_{\alpha s}$ diffuses along the membrane and activates the ion channel. In the second pathway $G_{\alpha s}$ activates AC which catalyses the conversion of ATP to cAMP. In turn cAMP binds to the inhibitor subunit of PKA, inducing a conformational change which allows PKA to break away in an active form. PKA then phosphorylates and activates the ion channel.

The co-immunoprecipitation blots revealed that in the unstimulated cells there is no apparent interaction between $G_{\alpha s}$ and the CACNA1C, this could be an indication that the channel has not been activated at this point. There is evidence of an interaction between $G_{\alpha s}$ and the KCNMA1 channel. After TNF stimulation both channels show interaction with $G_{\alpha s}$ and, although the interaction with the CACNA1C was only seen in one of the repeats, this is an indication that $G_{\alpha s}$ is interacting with these channels. After TSA stimulation both the KCNMA1 and the CACNA1C show an interaction with $G_{\alpha s}$. As has been explained previously, $G_{\alpha s}$ can both inhibit and stimulate the KCNMA1 channel and the switch between these two roles is thought to be regulated by changes in the properties of the KCNMA1 channel itself (Kume, Graziano *et al.* 1992). It is therefore possible that the effect of $G_{\alpha s}$ on the KCNMA1 channel could change depending on which splice variant of the KCNMA1 channel it is interacting with. This thesis has already demonstrated that TNF and TSA affect the expression of the KCNMA1 channel and its splice variants. This could then lead to the hypothesis that under TNF stimulation $G_{\alpha s}$ inhibits the KCNMA1 channel while under TSA stimulation $G_{\alpha s}$ stimulates the KCNMA1 channel.

Confirmation of these results and more investigation as to the mechanism by which TSA seems to promote $G_{\alpha s}$ stimulation of the CACNA1C is necessary. TSA is a broad spectrum

lysine de-acetylase inhibitor and as such it prevents the removal of lysine acetyl groups from a range of cellular proteins. $G_{\alpha s}$ has been shown to stimulate the CACNA1C via the cAMP/PKA pathway, and therefore TSA may promote this interaction via the maintenance of acetylation of key proteins in this pathway.

5.9.2 Cav-1 Interactions

Cav-1 is a scaffolding protein within the caveola complex and as such forms strong stable interactions with a variety of proteins (Thomas and Smart 2008). Caveolae, and hence cavolin, are thought to have a role in calcium signalling, through their ability to house key calcium signalling molecules within caveolae microdomains (Darby, Kwan *et al.* 2000, Chanrachakul 2006). This arrangement allows efficient signalling between the ion channels and their downstream targets.

The KCNMA1 channel has been shown to interact with Cav-1 via a key caveolin binding motif located between amino acids 1007-1015 within the C-terminal region (Alioua, Lu *et al.* 2008). This association of the KCNMA1 channel with Cav-1 is thought to have a negative effect on the gating properties of the KCNMA1 channel, as disruption of caveolae has been shown to increase KCNMA1 current (Lu, Alioua *et al.* 2006) this has been shown to be due a reduction in the surface expression of KCNMA1 when it is associated with Cav-1 (Alioua, Lu *et al.* 2008). However, this reduction in surface expression of the KCNMA1 channel may not equate to a removal of the KCNMA1 channel from either the plasma membrane or caveolae. Studies into the function of caveolae have demonstrated that they can open and close this means they can further regulate the surface expression of the KCNMA1 channel while it is still housed within the caveolae (Oh, McIntosh *et al.* 1998). Briefly, association of the KCNMA1 channel with Cav-1 promotes the localisation of the KCNMA1 channel to caveolae (Brainard, Miller *et al.* 2005). Once housed within the caveolae, surface expression of the KCNMA1 channel can then be regulated by the opening and closing of the caveolae. Taking this into account the observations by Lu *et al.* that the disruption of caveolae increases KCNMA1 current, could be explained by the disruption causing the caveolae to open hence increasing the surface expression of the KCNMA1 channel and subsequently increasing the KCNMA1 current. The observation by Alioua *et al.* could be interpreted as the KCNMA1 channels being housed in closed caveolae. Being able to regulate KCNMA1

surface expression in this way would allow rapid changes in KCNMA1 current depending on the needs of the cell.

Studies demonstrating the association between the CACNA1C and caveolae has mostly been undertaken in cardiac myocytes. In this cell system there appears to be a separation between CACNA1C which are involved in excitation-contraction coupling and those which are localised to caveolae and have a role in calcium signalling. For example it has been shown that caveolae-localised CACNA1C can selectively signal to the nucleus via the NFAT transcription factor, when this signalling is blocked there is no effect on the contractility of the cell (Makarewich, Correll *et al.* 2012). Moreover, in airway smooth muscle, CACNA1C have also been seen to localise within caveolae however the effect of this co-localisation on the contractility of the cell or on the kinetics of the CACNA1C gating were not examined (Darby, Kwan *et al.* 2000). A further study, within intestinal smooth muscle, again found CACNA1C to be localised within caveolae, however, unlike in cardiac muscle, this localisation is thought to have a role in contraction, relaxation, the production of calcium 'sparks' and possibly calcium waves (Daniel, El-Yazbi *et al.* 2006). This research would indicate that the localisation of CACNA1C within caveolae serves different functions within different tissues. However, these three studies were undertaken in different tissues and utilised different methodologies so it is difficult to determine if the observations are from distinct processes or whether each study has highlighted an aspect of a larger more complicated process. Ideally the techniques in each individual study should be utilised together in order to get a more complete picture of the role of the interaction between Cav-1 and CACNA1C.

Cav-1 forms covalent interactions with other proteins and the presence of these more stable protein interactions has meant that the co-immunoprecipitation data is much more consistent than was seen with the $G_{\alpha s}$ co-immunoprecipitation data. As discussed above association with Cav-1 was seen to reduce KCNMA1 cell surface expression (Lu, Alioua *et al.* 2006), although this may be via its association with closed caveolae, as Cav-1 has also been shown to target the KCNMA1 channel to caveolae (Brainard, Miller *et al.* 2005). A second site in the C-terminal region of the KCNMA1 channel has been shown to be important for plasma membrane localisation of the channel, this is located between amino acids 1117-1123 so it is possible that the KCNMA1 channel could still be transported to the membrane but that it is subsequently housed outside of the caveolae. If

this is the case then a lack of association with Cav-1 would increase surface expression of the KCNMA1 channel, whilst an association with Cav-1 would increase the localisation of the KCNMA1 channel to caveolae. As explained previously housing KCNMA1 channels within caveolae results in the decreased surface expression on the KCNMA1 channels (Alioua, Lu *et al.* 2008).

It is difficult to interpret the effect of these changes in association between the KCNMA1 and Cav-1 without further research into the associated changes in contractility, KCNMA1 protein localisation and KCNMA1 splice variant expression.

There is an association between CACNA1C and Cav-1 after TNF stimulation. This association could improve the efficiency of calcium signalling and excitation-contraction coupling and hence promote contraction.

5.9.3 β_2AR Interactions

The β_2AR is one of a family of adrenergic receptors and this family is among the best characterised of the G-protein coupled receptors. The adrenergic receptor family can act as scaffolding proteins and in this way they can bring together a variety of proteins and the proteins housed in this complex will then determine what cellular effect is seen upon activation of the adrenergic receptor (Cotecchia, Stanasila *et al.* 2012).

The KCNMA1 channel has been shown to be linked to the β_2AR and to mediate β_2AR activated relaxation. The mediation of relaxation has been shown to both cAMP dependent via PKA phosphorylation and cAMP independent possibly through direct action by $G_{\alpha s}$ independent of PKA-mediated phosphorylation (Kume, Graziano *et al.* 1992, Scornik, Codina *et al.* 1993, Kume, Hall *et al.* 1994, Tanaka, Yamashita *et al.* 2003, Tanaka, Horinouchi *et al.* 2005).

β_2AR agonists have been used to treat preterm labour. The caveat of these drugs, however, is that the longer they are administered the less effective they are at stopping contractions. This is due to the phenomenon of receptor internalisation which results in desensitization (van Geijn, Lenglet *et al.* 2005)

The CACNA1C has also been shown to be regulated by the β_2AR, in cardiac myocytes (Jurevicius and Fischmeister 1996, Schröder and Herzig 1999). However, unlike the KCNMA1 channel this regulation is completely cAMP-dependant via PKA phosphorylation (Jurevicius and Fischmeister 1996, Kuschel, Zhou *et al.* 1999, Schröder and Herzig 1999). Direct interaction between the CACNA1C and the β_2AR has also been reported in the brain, this complex was shown to be housed in the plasma membrane but did not associate with Cav-1 or Cav-3 indicating that this complex exists separately from caveolae (Davare, Avdonin *et al.* 2001). The regulation of the CACNA1C by the β_2AR within rat ventricular myocytes has been shown to be highly localised to the cell membrane and the signal did not diffuse through the cytosol (Chen-Izu, Xiao *et al.* 2000). This demonstrates that the β_2AR needs to be co-localised with the CACNA1C in order to be able to regulate it.

The data shown herein demonstrates that both the KCNMA1 and CACNA1C channels associate with the β_2AR in un-stimulated cells and following both TNF and TSA stimulation. This is a good indication that both channels are regulated by the β_2AR under these conditions. However neither TNF nor TSA had any detectable effect on the level of these associations.

The CACNA1C was also shown to associate with a 42kDa β_2AR protein, although the level of association was unchanged by the different stimulations. Salahpour *et al.* demonstrated that homodimerisation of the β_2AR was a prerequisite for cell surface trafficking (Salahpour, Angers *et al.* 2004). This would then suggest that the 42kDa β_2AR has either been internalised or retained in the ER. Receptors that fail to reach the plasma membrane have been shown to be capable of exerting a dominant negative effect on the trafficking of other receptors to the membrane (Lan, Kuravi *et al.* 2011). It would interesting to elucidate if the association between the 42kDa β_2AR and the CACNA1C is a mechanism by which the CACNA1C can be retained intracellularly or whether this localisation is co-incidental due to both proteins being independently retained in the ER. The later could putatively occur if the β_2AR failed to dimerise and the CACNA1C channel expressed the Mk44 +132bp variant which has been shown to result in the C-terminus being localised to the ER.

5.9.4 Channel Interactions

The KCNMA1 channel has been shown to undergo proteolytic cleavage and as a result the N-terminal localises to the plasma membrane while the C-terminal localises to the endoplasmic reticulum (Korovkina, Brainard *et al.* 2006). The C-terminal can then non-covalently re-associate with the N-terminal domain following calcium release from the sarcoplasmic reticulum. Association of the N- and C-termini results in a decrease in calcium and voltage sensitivity of the KCNMA1 channel. This may be a result of increased interaction with Cav-1 which is dependent on the C-terminal region (Alioua, Lu *et al.* 2008). The data described herein reveals an association between the KCNMA1 N- and C-termini which could result in the decreased calcium and voltage sensitivity of the KCNMA1 channel which would result in a more contractile phenotype.

As with the KCNMA1 channel the CACNA1C channel has also been shown to undergo proteolytic cleavage. Following this cleavage the cleaved CACNA1C C-terminal can non-covalently associate with the rest of the channel negatively affecting channel gating. The IP data from the CACNA1C N-terminal IP followed by western blotting with the CACNA1C C-terminal antibody reveal a 240kDa in the un-stimulated and both a 240 and a 190kDa band in the un-stimulated and TNF stimulated groups (Figure 5.7B). This would lead to idea that the 190kDa protein could be formed in an alternative way through use of alternative splice variants possibly at splice site L10. These splice variants alter the length of the linking loop between IV S2 and IV S4 in the channel. A shorter linking loop, which would need to be utilised to form the shorter channel, results in a CACNA1C with decreased excitation-contraction coupling and ultimately a less contractile phenotype. This would result in a shorter channel containing both the N- and C-terminii as detected on the blots. It would be necessary to sequence this shorter form of the protein to determine if the truncation is as a result of differences in the length of the linking loop between IV S2 and IV S4 or as a result of the cleavage of the C-terminal domain. Folllowing TSA stimulation a single band was seen, this band was between 240 and 190kDa (Figure 5.7B) and therefore may be representative of the 190kDa proteolytically cleaved CACNA1C channel with the C-terminal region non-covalently associated with it.

The KCNMA1 C-terminal was also found associated with the CACNA1C N-terminal in un-stimulated samples and following exposure to either TNF or TSA (Figure 5.7A). This

association was seen both with full length KCNMA1 channel and with the 65kDa KCNMA1 channel which lacks the N-terminal domain (Figure 5.7A). As explained above the C-terminal domain can be cleaved from the body of the channel and can then non-covalently re-associate with the N-terminal domain. Therefore the interaction between the 65kDa KCNMA1 protein and the CACNA1C may demonstrate that the N- and C-termini of the KCNMA1 channel are non-covalently associated. This non-covalent association can be lost during the immunoprecipitation procedure and hence the results show the KCNMA1 C-terminal domain is associated with CACNA1C N-terminal. Alternatively, it is possible that the CACNA1C N-terminal is interacting only with this C-terminal domain without any link to the N-terminal domain of the KCNMA1 channel.

The CACNA1C C-terminal region was found associated with the KCNMA1 N-terminal following exposure to either TNF or TSA (Figure 5.7B). Following TNF stimulation this association was between the KCNMA1 N-terminal domain and the 190kDa CACNA1C protein which is lacking its C-terminal domain. This isoform of the CACNA1C has four to six fold higher ion conductance than the longer 240kDa isoform (Gao, Cuadra *et al.* 2001). Association between the KCNMA1 channel and this 190kDa isoform may permit the KCNMA1 channel to respond more rapidly and hence limit the effect of the increased ion conductance of the 190kDa CACNA1C. Following TSA stimulation this association was between the KCNMA1 N-terminal and a CACNA1C channel between 240 and 190kDa (Figure 5.7B) and therefore may be representative of the 190kDa proteolytically cleaved CACNA1C channel with the C-terminal region non-covalently associated with it.

5.10 Conclusion

Although the KCNMA1 and CACNA1C were seen to associate with each other, $G_{\alpha s}$, Cav-1 and β_2AR, it is difficult to determine from the blotting data if this association is representative of close proximity to each other on the membrane or if they are housed together either in a complex with the β_2AR or within caveolae or both. This is a limitation of the co-immunoprecipitation technique as it cannot identify simultaneously all the proteins within a complex. It also does not provide evidence of direct protein:protein interactions. Positive results therefore, may indicate that two proteins interact directly or may indicate that they interact via one or more bridging molecules. This could include

bridging proteins, nucleic acids (DNA or RNA), or other molecules. To determine the full complement of proteins with the putative complexes discussed above it would be necessary to carry out triple staining and confocal microscopy analysis, and this is something which should be done in the near future. To identify direct interaction between the proteins methods like protein affinity chromatography, or affinity blotting could be utilised, ion exchange column could then be used to determine the strength of this binding.

In the preceding chapters of this thesis it has been demonstrated that the myometrial cell cultures express a range of KCNMA1 channel, CACNA1C and CACNA1G splice variants. Alongside, and perhaps as a result of, these different splice variants they also have been shown to express a range of different protein isoforms and protein fragments, which may be generated as a result of proteolytic cleavage. It has been demonstrated that these different protein isoforms and fragments are localised to both the cytoplasm and plasma membrane. In addition, it has been demonstrated that the KCNMA1 and CACNA1C channels associate with each other as well as $G_{\alpha s}$, Cav-1 and β_2AR.

Chapter 6: Regulation of Transcription

6.1 Introduction

In Chapter three the expression of the KCNMA1, CACNA1C and CACNA1G channels was examined to determine if the contractile effect of TNF or the relaxatory effect of TSA was mediated via changes in the amount of channels expressed or in the splice variant profile of the expressed channels. The results of these studies indicated that in the model i.e. in the un-stimulated samples, there were a number of different splice variants being expressed by the channels. These included the KCNMA1 Mk44+132bp, Mk44-132bp, M2 (exon 11+12), M3 (exon 19), M4 (STREX2) and M5 (exon 29) variants, the CACNA1C L4 (exon 8* or Exon 8), L10 (exons 30, 31, 33 and 34 or exons 30, 31, 32 and 34) and L11 (40, 41, 42 and 43) variants and the CACNA1G T2 (exon 14 deleted), T4 (exon 25a), T6 (exon 26) and T7/8 (exon 31A or exon 30B and 31A) variants. Upon TNF stimulation, expression of both the KCNMA1 and CACNA1C channel mRNA was significantly up-regulated. Moreover, upon TSA stimulation, both channels were significantly down-regulated and the splice variant profile of the CACNA1C and CACNA1G was significantly altered. Consequently, the next step was to examine how these changes in channel expression were regulated at the transcriptional level.

In vivo, DNA is found in complex with histones and other ancillary proteins: this complex is termed chromatin. The purpose of chromatin is to package the DNA into a small enough volume to fit in the nucleus while protecting the DNA structure and sequence. This packaging of the DNA also has a role in controlling gene expression. Heterochromatin is a tightly packed form of chromatin which can silence gene transcription. Euchromatin is less condensed and contains the majority of actively transcribed genes.

The basic unit of chromatin is the nucleosome, this consists of 147bp of DNA wrapped 1.65 times round an octamer of histones which contains two copies of each histone protein: H2A, H2B, H3 and H4. Within this complex the DNA has 14 contact points with the histone octamer (Luger, Mäder *et al.* 1997), this forms a stable protein:DNA complex. Although this is a stable complex it is also dynamic.

These nucleosomes have been shown to impede transcription (Knezetic and Luse 1986, Lorch, LaPointe *et al.* 1987), and so to enable transcription to take place a number of modification of this nucleosome complex occur.

The histones themselves can undergo modifications including acetylation (Bannister and Kouzarides 1996, Greer and Shi 2012), methylation (Rea, Eisenhaber *et al.* 2000, Fuks, Hurd *et al.* 2003, Fuks, Hurd *et al.* 2003, Levy, Kuo *et al.* 2011, Greer and Shi 2012, Yang, Guo *et al.* 2012) and phosphorylation (Hurd, Bannister *et al.* 2009) all of which are laid down in a dynamic fashion and can potentially be inherited during meiosis (Greer and Shi 2012). Histone acetylation is the covalent modification of the epsilon-amino group of conserved lysines by histone acetyltransferases. Histone acetylation was first observationally linked to transcription by Allfrey *et al.* (Allfrey, Faulkner *et al.* 1964). Since this early observation a large amount of research has focused on this link between acetylation and transcription (Brownell, Zhou *et al.* 1996, Candau, Zhou *et al.* 1997, Kuo, Zhou *et al.* 1998) and has strengthened the hypothesis that histone acetylation is an important part of transcriptional activation. Conversely, histone deacetylation, the process by which the acetylation is removed from the lysine by histone deacetylases (HDACs) has been linked to transcriptional repression (Taunton, Hassig *et al.* 1996, Yang and Seto 2003). In the de-acetylated state the tail of the histone proteins have a positive charge and this promotes interaction with the negatively charged phosphate groups of the DNA, when the tail is acetylated the charge changes from positive to neutral and this disrupts the histone:DNA interaction. This weaker association has the result of making the DNA more accessible to transcription factors (Reinke and Hörz 2003, Zhao, Herrera-Diaz *et al.* 2005).

The first step in the initiation of transcription is formation of the preinitiation complex, containing Transcription Factor (TF) IIB, TFIID, TFIIE, TFIIF and TFIIH. These assemble on the promoter DNA with RNA polymerase II and support the accurate initiation of transcription (Nikolov and Burley 1997). Following the formation of this complex, further transcription factors are then recruited to the promoter these transcription factors bind to DNA in a sequence-specific manner. Once bound to the DNA they can either promote or block the action of RNA polymerase II, which controls the transcription of the gene, and in this way can enhance or reduce the expression of specific genes. These transcription factors can be either constitutive or inducible. Constitutive transcription factors are continually expressed, and their expression is not dependent on extracellular signals; on the other had inducible transcription factors are synthesised within the cell in response to certain stimuli. One such stimuli which can induce nuclear

localisation of transcription factors is TNF. This may be the mechanism by which TNF regulates the expression of the channels.

Another factor which can affect the transcription of a gene is DNA methylation. This is a biochemical process which adds a methyl group to either the fifth carbon atom of cytosine nucleotides or the sixth nitrogen atom of adenine nucleotides. In mammals approximately 60-90% of all CpGs are methylated, the un-methylated CpGs are often clustered in CpG islands which are located in the regulatory regions of gene (Deaton and Bird 2011). Abnormal methylation of these CpG islands can result in the gene being transcriptionally silenced. There are two methods by which methylation can affect transcription, the first is by the methyl group physically hampering transcription factor binding. Secondly, methylated DNA can be bound by proteins termed, methyl-CpG-binding domain proteins, these proteins then recruit other proteins such as histone deacetylases, which then modify the histones resulting in the formation of tightly packed heterochromatin which is inaccessible to transcription factors. Increased expression of pro-inflammatory cytokines, including TNF, have been shown to mediate inflammation-induced DNA methylation changes (Hahn, Hahn *et al.* 2008, Niwa, Tsukamoto *et al.* 2010, Hur, Niwa *et al.* 2011, Kominsky, Keely *et al.* 2011, Katsurano, Niwa *et al.* 2012) and therefore this may be an alternative method by which TNF can regulate the transcription of the channels.

Although there has been some research indicating that hormones such as estrogen, progesterone (Holdiman, Fergus *et al.* 2002, Zhu, Eghbali *et al.* 2005), and insulin (Davies, Zhao *et al.* 2007) can regulate the expression of the KCNMA1, CACNA1C and CACNA1G channels or their splice variants, there is very little research into how this regulation occurs at the level of chromatin:protein interactions. This is a significant void in the understanding of how these channels are regulated and in turn how they regulate the contractility of the myometrium. If the aim is to be able to manipulate the expression of the channels and their splice variants in order to directly modulate the contractility of the myometrium it is vitally important to fill in this void in our understanding of how the channels are regulated.

6.2 Identification of Transcription Factor Binding Sites

For each of the channels the region encompassing the promoter regions of the genes, was identified and the sequence entered into the TransFac® database (Matys, Kel-Margoulis *et al.* 2006). Transfac® provides data on eukaryotic transcription factors, their experimentally proven binding sites and consensus sequences (http://www.gene-regulation.com/pub/databases.html). Using the TFsearch tool the genomic sequences were interrogated to identify putative transcription factor binding sites. From the list of transcription factor binding sites identified, a number of transcription factors were selected for further analysis (Figure 6.1).

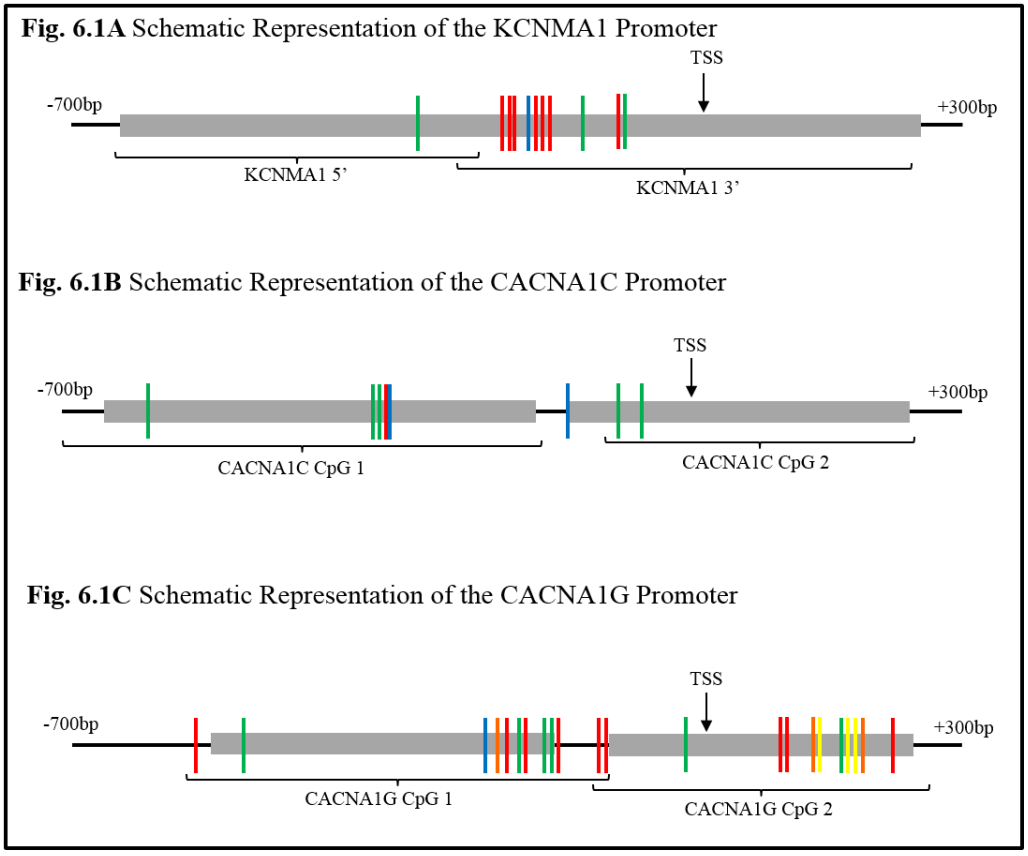


Figure 6.1 Schematic Representation of the KCNMA1, CACNA1C and CACNA1G Promoters
(A) KCNMA1 promoter; **(B)** CACNA1C promoter and **(C)** CACNA1G promoter. The transcription start site (TSS) is indicated by a black arrow. CpG islands identified within the promoters are represented by the grey bar. Transcription factor binding motifs are indicated by vertical bars, green represents SP1 motifs, Red indicates MZF1 motifs, Blue indicates CBP motifs, Orange indicates p50 motifs and Yellow indicates RelA motifs.

NFκB has been implicated in the regulation of all three channels (Furukawa and Mattson 1998, Green and Peers 2002, Scragg, Fearon *et al.* 2005, Cookson, Waite *et al.* 2015). Although, binding sites for RelA, cRel and p50 were identified within the CACNA1G promoter, NFκB binding sites were not identified from the genomic sequence of either

the KCNMA1 or CACNA1C promoters. However, it was decided to perform ChIPs on all three promoters with both RelA and p50 antisera as the lack of NFκB consensus binding site may indicate that within these promoters NFκB is binding to a less well described non-canonical motif (Wong, Teixeira *et al.* 2011).

6.3 Identification of CpG Islands Within the Promoter Regions of the Channel Genes

The region encompassing the promoter regions of the genes, was found to be GC rich. The sequence of these regions was entered into the MethPrimer database (Li and Dahiya 2002) for CpG island prediction. The following parameters were used for CpG island prediction: window size was set to 100, so the program will calculate parameters within this window, the step was set to 1, the observed-to-expected GC ratio was set to 0.6 and the percentage of G plus C was set to 50%. Briefly the program will examine 100bp at once, this is termed the window, then it will move along one bp, termed the step, and examine 100bp from this location. Within each window the program will calculate the observed number of CpGs within the window in comparison to the expected number of CpGs. Finally within each window the program will calculate the total number of C and G and express this as a percentage of the total number of bp in the window. The formal definition of a CpG island is a region with an observed to expected ratio of >60%, meaning 60% of the G and C in the sequence are in the form of CpG, and a GC percentage >50%, meaning more than 50 of the nucleotides in the window are either C or G.

Within the KCNMA1 promoter a single CpG island encompassing 898bp (653bp upstream to 244bp downstream of the TSS) was identified (Figure 6.1A). There were two CpG island identified within the CACNA1C promoter sequence: the first encompassing 480bp (654bp to 175bp upstream of the TSS) and the second encompassing 383bp (141bp upstream to 241bp downstream of the TSS; Figure 6.1B). Finally two CpG islands were also identified within the CACNA1G promoter sequence: the first encompassing 386bp (548bp to 163bp upstream of the TSS) and the second encompassing 342bp (96bp upstream to 245bp downstream of the TSS; Figure 6.1C).

6.4 ChIP / MeDIP Primer Design and PCR Optimisation.

Primers were designed to flank each of the CpG Islands predicted in the CACNA1C and CACNA1G promoters for use in the ChIP and MeDIP assays. Due to the predicted size of the CpG Island in the KCNMA1 promoter, it was decided to design primers sets to cover the 5' region and 3' region individually. The primer sequences, location and product size are listed in Chapter Two *Materials and Methods;* Table 2.4.

It was not possible to get PCR amplification utilising *Taq* polymerase for any of the promoter region primer pairs (data not shown) and the reason for this could not be defined. Therefore this lack of amplification could be due to a combination of factors including the GC-rich nature of both the promoter regions and primers and possibly the particular batch of *Taq* used had lower specific activity. However, the same *Taq* polymerase was utilised successfully for the PCR reactions detailed in chapter 3. Due to these difficulties, the Herculase II fusion polymerase was trialled. Herculase II fusion polymerase is specifically engineered to amplify difficult targets such as GC-rich regions. Consequently after optimisation, PCR amplification of the GC-rich regions was possible with all the primer sets.

For optimisation an initial annealing temperature of 5°C below the average T_m of the primer pair was selected. Then the optimisation parameters suggested by the Herculase II fusion polymerase manufacturer were used. Briefly, 0.25µl Herculase II Fusion polymerase (note that the manufacturer, Agilent, did not provide a value for the number of enzyme units/µl), 0.25µM each primer, 250µM each dNTP. Manufacturers' guidelines suggested DMSO should be trialled at between 0-8% (v/v) of the final concentration. Therefore optimisation PCRs were performed with 1%, 4% and 8% (v/v) DMSO. Initially the manufacturers' guidelines regarding PCR cycling conditions were also followed. Briefly, these were: 95°C for 2 minutes then 30 cycles of 95°C for 20 seconds, Primer T_m less 5°C for 30 seconds, 72°C for 20 seconds then a final extension at 72°C for 3 minutes.

Following the initial steps detailed above, further optimisation reactions were undertaken as needed for each of the individual primer sets. These steps included: increasing the number of cycles to improve the signal intensity, decreasing the annealing time or altering the annealing temperature to improve the specificity of primer binding, increasing the

elongation time to decrease the number of low molecular weight non-specific amplicons, decreasing the elongation time to decrease the number of high molecular weight non-specific amplicons and decreasing the amount of input DNA to reducing smearing. The specific optimisations undertaken for each primer set is summarised below

For the KCNMA1 3' primers, PCR amplification was successful with both 1% and 4% (v/v) DMSO (Figure 6.2A I). The reaction utilising 4% (v/v) DMSO had fewer non-specific PCR products and so it was decided to further optimise this reaction. The following parameters were altered, the number of cycles was increased to 34, the annealing time was decreased to 20 seconds and the elongation increased to 30 seconds. These adjustments proved successful and the non-specific PCR products were almost eliminated (Figure 6.2A II).

For the KCNMA1 5' primers, PCR amplification was most successful with 4% (v/v) DMSO. However, the specific band was very faint in comparison to non-specific amplicons (Figure 6.2B I). Therefore the annealing temperature was reduced by 1°C and the other parameters were unchanged. After this modification the specific band had brighter intensity but there was a smear on the gel (Figure 6.2B II). This may have been a reflection on too great an amount of input DNA, therefore, the input DNA was reduced to 1µl to try and eliminate the smearing, while all the other parameters remained the same. Reducing the input DNA reduced the smear but significant non-specific amplicons remained (Figure 6.2B III). To overcome this two further optimisation protocols were tried: firstly the number of cycles was increased to 38; secondly the elongation step was increased to 30 seconds. Again all other parameters were unchanged. Increasing the cycle number was not effective (Figure 6.2B IV). However, increasing the elongation time significantly reduced the non-specific PCR products (Figure 6.2B IV).

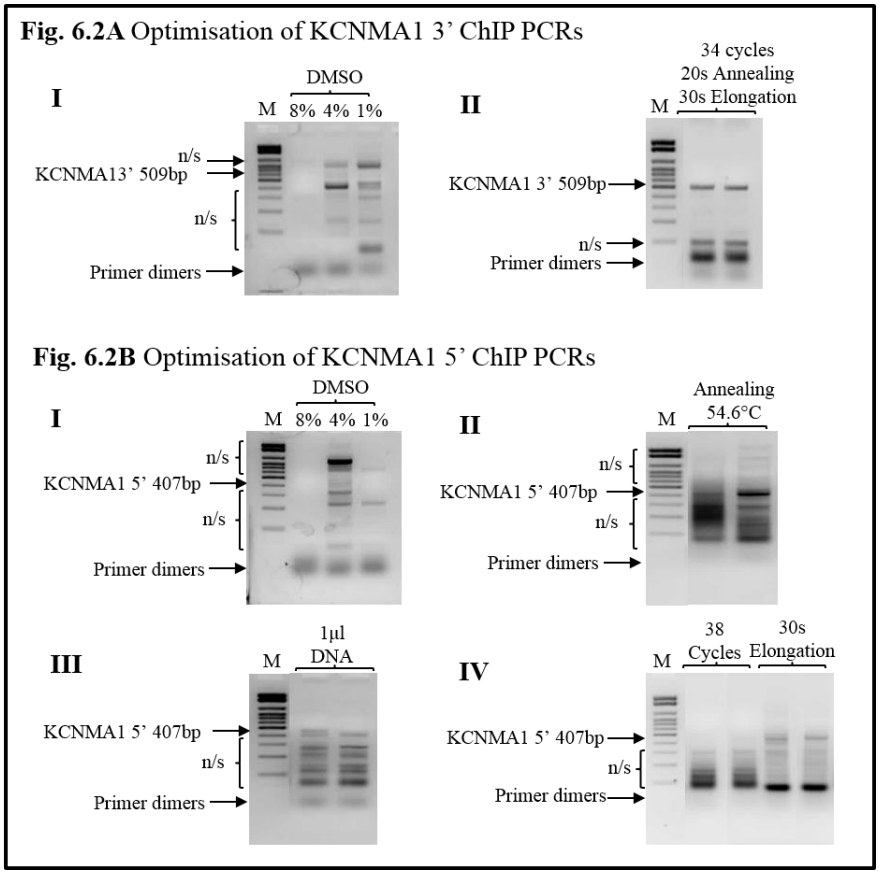


Figure 6.2 Optimisation of KCNMA1 Promoter ChIP PCRs
(A) Optimisation of KCNMA1 3' Primers. PCRs were performed with different concentrations of DMSO ((v/v) 1%, 4% and 8%) PCRs worked best with 4% (v/v) DMSO (I). PCRs were repeated with 4% (v/v) DMSO, 34 cycles, a 20 second annealing step and a 30 second elongation step; this PCR was successful (II). **(B)** Optimisation of KCNMA1 5' Primers. PCRs were performed with different concentrations of DMSO ((v/v) 1%, 4% and 8%) PCRs worked best with 4% (v/v) DMSO (I). PCRs were repeated with 4% (v/v) DMSO and an annealing temperature of 54.6°C (II). These PCRs were obscured by smearing on the gel. The previous PCR was repeated but with 1µl DNA (III). This improved the results but there was still non-specific bands. The previous PCR was repeated with either 38 cycles or a 30 second elongation step (IV). The PCR with a 30 second elongation step was successful.

For the CACNA1C CpG Island 1 primers, PCR amplification was successful with 4% DMSO, however there was smearing and non-specific banding also present (Figure 6.3A I). As with the KCNMA1 5' primers, a number of different optimisation steps were employed.

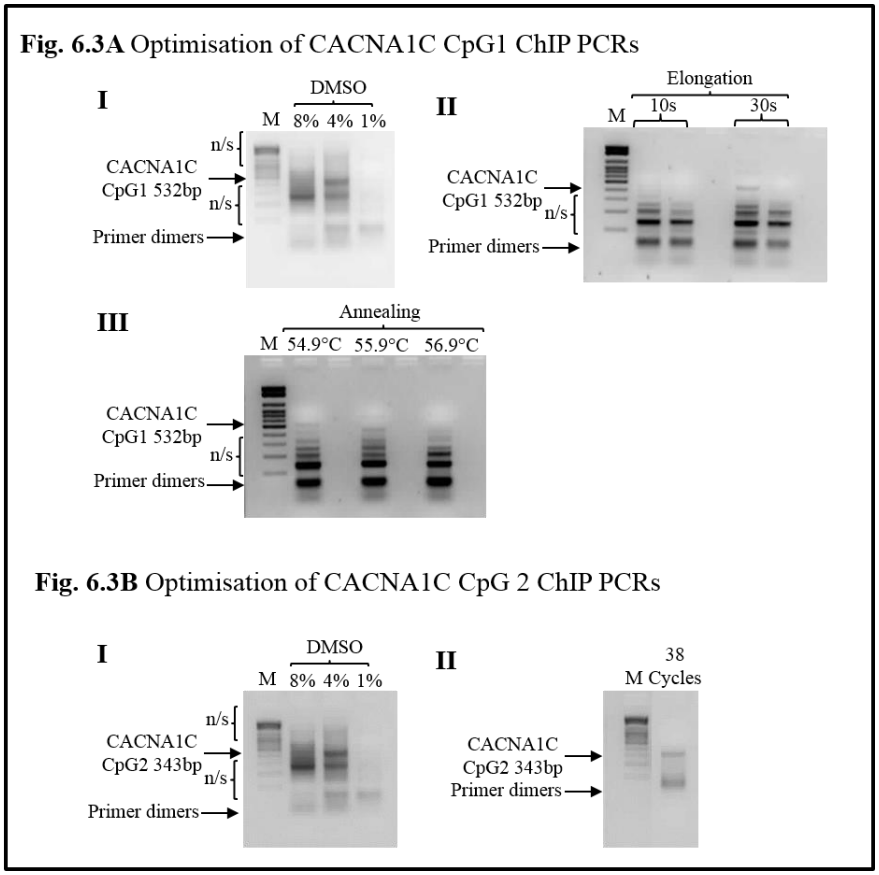


Figure 6.3 Optimisation of CACNA1C Promoter ChIP PCRs
(A) Optimisation of CACNA1C CpG 1 Primers. PCRs were performed with different concentrations of DMSO ((v/v) 1%, 4% and 8%) PCRs worked best with 4% (v/v) DMSO (I). PCRs were repeated with 4% (v/v) DMSO and with either a 10 second or a 30 second elongation step (II). The PCRs were improved by the 30 second elongation step. This PCR was repeated a three different annealing temperatures; 54.9°C, 55.9°C and 56.9°C (III). The PCR was successful at 54.9°C**. (B)** Optimisation of CACNA1C CpG 2 Primers. PCRs were performed with different concentrations of DMSO ((v/v) 1%, 4% and 8%) PCRs worked best with 4% (v/v) DMSO (I). PCRs were repeated with 4% (v/v) DMSO and 38 cycles (II), this PCR was successful.

Briefly, these included: reducing the DNA input to 1µl alongside either increasing the elongation to 30 seconds or decreasing the elongation to 20 seconds (Figure 6.3A II) and optimisation of the annealing temperature (Figure 6.3A III). The lowest annealing temperature (54.9°C) produced a band of the correct size although this was faint it was un-obscured by non-specific banding (Figure 6.3A III).

For the CACNA1C CpG Island 2 primers, PCR amplification was successful with 4% DMSO (Figure 6.3B I), however this band was faint. The cycle number was increased to 38 to improve the intensity of this band. This produced a clear band of the correct size without either smearing or non-specific bands (Figure 6.3B II).

For the CACNA1G CpG Island 1 primers, PCR amplification was successful with 1% DMSO, therefore no further optimisation was required (Figure 6.4A).

For the CACNA1G CpG Island 2 primers, PCR amplification was successful with 1% DMSO, therefore no further optimisation was required (Figure 6.4B).

Fig. 6.4A Optimisation of CACANA1G CpG 1 ChIP PCRs

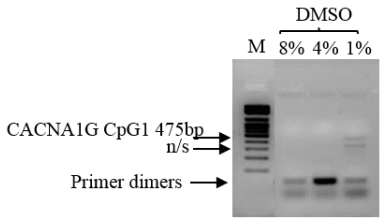


Fig. 6.4B Optimisation of CACNA1G CpG 2 ChIP PCRs

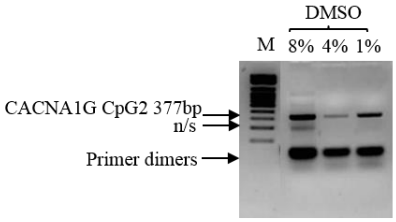


Figure 6.4 Optimisation of CACNA1G Promoter ChIP PCRs
(A) Optimisation of CACNA1G CpG 1 Primers. PCRs were performed with different concentrations of DMSO ((v/v) 1%, 4% and 8%) PCRs worked with 1% (v/v) DMSO. **(B)** Optimisation of CACNA1G CpG 2 Primers. PCRs were performed with different concentrations of DMSO ((v/v) 1%, 4% and 8%) PCRs worked best with 1% (v/v) DMSO.

6.5 Experimental Results

6.5.1 The ChIP Assay

In order to study the transcription of the KCNMA1, CACNA1C and CACNA1G channels the ChIP assay was used. From the TransFac analysis of the promoter regions a number of transcription factors were selected for study utilising the ChIP assay. These were: CBP, MZF, SP1, H3, p50 and RelA. Briefly, six T-75 flasks of primary myocytes were grown to 100% confluence. Two of these flasks were then stimulated with 10 ng/ml TNF for 1 h, two were stimulated with 100 ng/ml TSA for 24hrs and two were left unstimulated. The ChIP assay was then carried out as detailed in Chapter 2 *Materials and Methods*.

6.5.2 The MeDIP Assay

The MeDIP assay is a modified version of the ChIP assay, instead of isolating chromatin which is bound by specific transcription factors, the MeDIP assay selectively isolates methylated genomic DNA sequences. It does this by utilising an antibody specific for 5-methylcytosine. Six T75 flasks of primary myocytes were grown to 100% confluence. Two of these flasks were then stimulated with 10ng/ml TNF for one hour, two were stimulated with 100ng/ml TSA for 24 hours and two were left unstimulated. The MeDIP assay was then carried out using the EpiQuik™ Methylated DNA Immunoprecipitation Kit as detailed in Chapter 2 *Materials and Methods* section 2.8

6.6 Analysis of Data

After manual band quantification of the PCR images, analysis was performed as follows: the value ascribed to nonspecific binding of IgG was subtracted from the specific binding value. That value was then expressed as a percentage of the input fraction with the input fraction intensity being taken as 100%. All experiments were repeated three times and the results are expressed as the mean ± SD. Each repeat represented a single myometrial biopsy. All data analyses were conducted on GraphPad Prism version 6, where a one way ANOVA with Dunett's post-test was performed to compare the individual stimulations against the unstimulated control; $p<0.05$ was considered statistically significant.

6.6.2 DNA Shearing

The importance of correctly shearing the DNA for use in the ChIP and MeDIP assays cannot be overstated. Over shearing the DNA will result in very small promoter fragments which can be detrimental to the downstream PCR analysis. This phenomenon occurs because very small fragments (<100bp) can reduce primer recognition and hence reduce PCR efficiency. Moreover, over-shearing the DNA can damage the transcription factor proteins and hence reduce the antibody binding efficiency thus compromising the immunoprecipitation. Under-shearing the DNA is also problematic. This produces large fragments which can result in the immunoprecipitation of chromatin distant from the transcription factor binding site and hence increase the risk of false positive results. After sonication, a portion of the chromatin from each sample was analysed by AGE to determine all the samples had fragments within the correct range (200 – 1000bp; Figure 6.5).

6.6.3 ChIP Controls

A Histone H3 antibody was used as a control for the ChIP assay as the amount of H3 associated with a promoter should be relatively consistent across all stimulations. Significant variations in the normalised H3 enrichment would indicate significantly different cell numbers in the different ChIP samples. Significant differences in cell numbers may skew the results of the ChIP assay. Following the ChIP assay, when the promoter primer PCRs were subsequently performed on the H3 immunoprecipitated chromatin, the results were inconsistent (Figure 6.5B). Therefore, no further analysis of these was undertaken

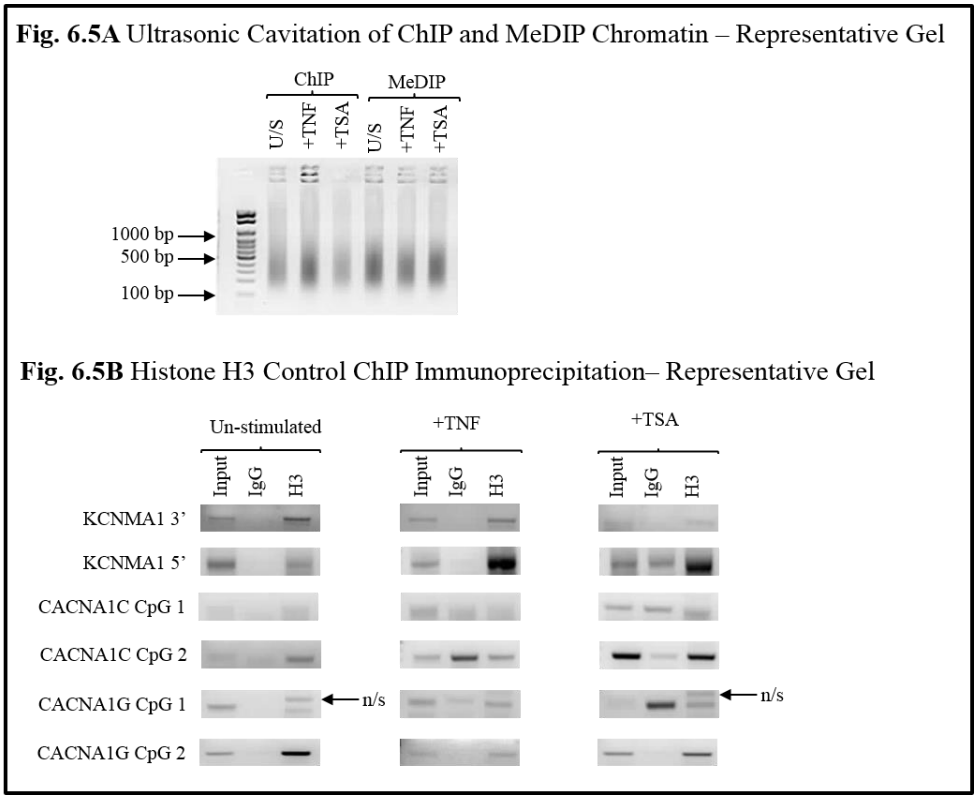


Figure 6.5 ChIP Assay Controls
(A) Ultrasonic Cavitation of Myometrial Cell DNA. Formaldehyde fixed nuclei isolated during the ChIP and MeDIP assays were sonicated on ice for 3 x 10 second intervals. The sheared DNA was visualised on a 1.5% (w/v) agarose gel and calibrated against a LowRanger ladder (M). The DNA shears were most intense between 500 and 200bp**. (B)** Histone H3 Conrol ChIP Immunoprecipitation – Repesentative gels. ChIP chromatin was immunoprecipitated using Histone H3 antisera or non-specific rabbit IgG, the resulting chromatin was then amplified using the channel promoter specific primers.

6.6.4 Validation of ChIP Protocol

The difficulties in generating consistent results which were experienced during the ChIP and MeDIP assays could be due to a number of factors. Firstly the ChIP and MeDIP assay themselves may have been poorly executed and hence the inconsistency between the repeats. Secondly, the high GC content of the promoter regions under study may be affecting the efficiency and hence the consistency of the PCR reactions. ChIP has been routinely carried out within the research group utilising RelA antiserum and a robust positive control for this ChIP is available. Therefore, this positive control PCR was performed utilising the same ChIP chromatin immunoprecipitated with RelA as was used for the PCRs above. This will reveal if the ChIP assay has worked as expected. The positive control IκBα PCR worked well with minimal IgG signal and consistent strong input and specific signal intensity (Figure 6.10). This indicates that the problems experienced are likely due to the GC rich nature of the promoter regions.

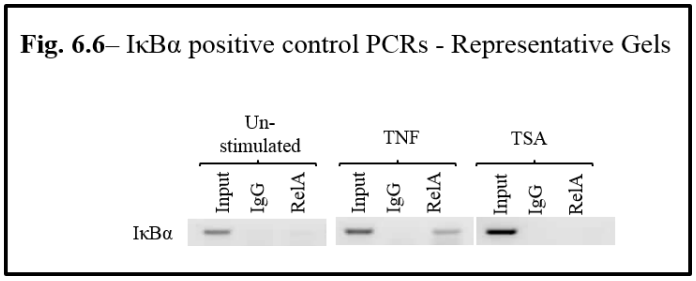


Figure 6.6 IκBα Positive Control ChIP PCR
ChIP chromatin was immunoprecipitated using RelA or non-specific rabbit IgG, the resulting chromatin was then amplified using primers specific to the IκBα promoter. The IgG signal intensity is low, indicating that there is little non-specific banding. There is a low level of Rel A binding in the un-stimulated sample, TNF results in an increase in the signal intensity for RelA binding and TSA results in the loss of RelA binding. These results indicate that the ChIP assay is working correctly.

6.7 Transcription Factor Binding

Despite the optimisation of the promoter primer PCRs described above the ChIP PCRs were repeatedly inconsistent, displaying a high degree of variability in the input, IgG and specific signal intensities. This made analysis difficult and any meaningful interpretation of the analysis impossible. A high level of IgG binding was observed in number of the ChIP reactions (Figure 6.6A I, B I and 6.8 A 1). The intensity of the IgG band is a measure of the level of non-specific binding within the specific IP reaction. Therefore, the intensity of the IgG bands observed in the figures listed above, would suggest that there is a high level of non-specific binding in these ChIP reactions. The IgG samples underwent the same PCR amplification as the specific IP samples and, as stated above, also suffered

from the same inconsistencies. It is therefore, possible that these IgG bands do not accurately reflect the level of background signal. Therefore, figures are only shown for gels were the intensity of the specific band is greater than that for the IgG and only simple observations from the ChIP PCRs are discussed below.

6.7.1 The KCNMA1 Promoter

6.7.1.1 The KCNMA1 Promoter 3' Region

In the un-stimulated sample SP1, CBP. MZF (Figure 6.7 A I) and RelA (Figure 6.7 A II) were observed to occupy this region of the promoter. Following exposure to TNF, the binding observed in this region of the promoter was unchanged (Figure 6.7 A I and II). However, following exposure to TSA no binding was observed for MZF (Figure 6.7 A I) or RelA (Figure 6.7 A II) on the 3' end of the promoter.

Fig. 6.7A – KCNMA1 3' ChIP Immunoprecipitation PCRs - Representative Gel

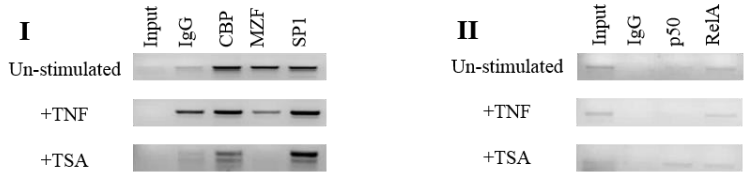


Fig. 6.7B – KCNMA1 5' ChIP Immunoprecipitation PCRs - Representative Gel

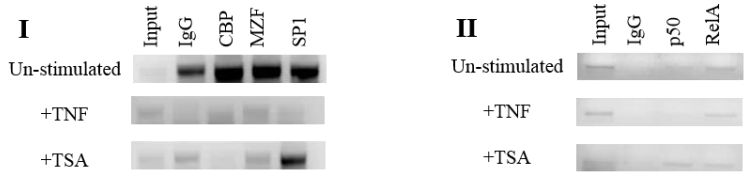


Figure 6.7 KCNMA1 Promoter ChIP PCRs - Representative Gels
ChIP chromatin was immunoprecipitated using CBP, MZF, SP1 (I), p50, RelA (II) or non-specific rabbit IgG, the resulting chromatin was then amplified using either the KCNMA1 channel 3' promoter specific primers **(A)** or the KCNMA1 channel 5' promoter specific primers **(B).** The IgG signal intensities varied greatly between repeats and between the different PCRs. Specific signal within the IP lane also varied greatly. No further analysis was undertaken.

6.7.1.1 The KCNMA1 Promoter 5' Region

In the un-stimulated sample SP1 CBP, MZF (Figure 6.7 B I) and p50 (Figure 6.7 B II) were observed to occupy this region of the promoter. Following exposure to either TNF

or TSA, the binding observed in this region of the promoter was visually decreased (Figure 6.7 B I and II).

6.7.2 The CACNA1C Calcium Channel Promoter

6.7.2.1 The CACNA1C Promoter CpG1 Region

In the un-stimulated sample SP1 CBP, and MZF were observed to occupy this region of the promoter (Figure 6.8 A). Binding was not observed for either p50 or RelA, these PCRs also had high levels of IgG signal. Exposure to either TNF or TSA did not appear to affect the binding of SP1, CBP or MZF (Figure 6.8 A). Exposure to TSA did result in the observation of RelA and p50 binding to this region of the promoter, however these PCRs also had high levels of IgG signal and so little confidence can be placed in the specificity of the RelA or p50 bands.

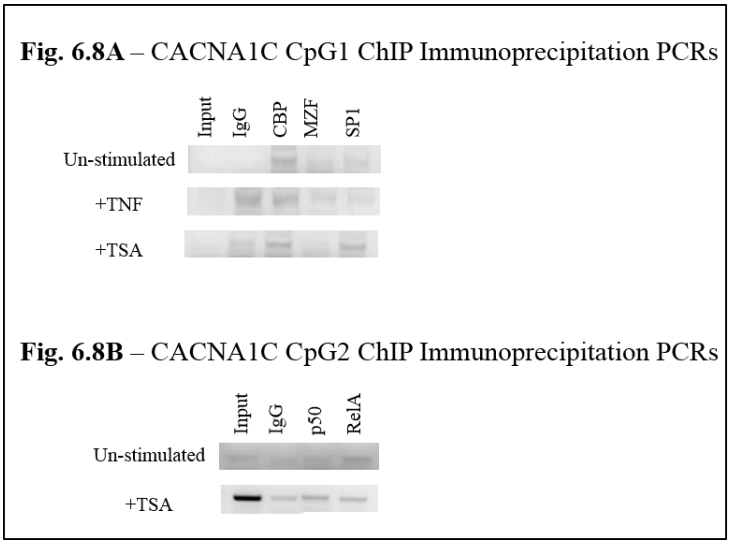


Figure 6.8 CACNA1C Promoter ChIP PCRs - Representative Gels
ChIP chromatin was immunoprecipitated using CBP, MZF, SP1, p50, RelA or non-specific rabbit IgG, the resulting chromatin was then amplified using either the CACNA1C CpG1 promoter specific primers **(A)** or the CACNA1C CpG2 promoter specific primers **(B).** The IgG signal intensities varied greatly between repeats and between the different PCRs. Specific signal within the IP lane also varied greatly. No further analysis was undertaken.

6.7.2.2 The CACNA1C Promoter CpG2 Region

In the un-stimulated sample SP1 CBP, and MZF were observed to occupy this region of the promoter, intense IgG was also observed in these ChIPs. Unlike the CACNA1C CpG1 region binding was also observed for RelA (Figure 6.7 B). Exposure to either TNF or TSA did not appear to affect the binding of SP1, CBP or MZF, however, these ChIPs

again showed a high level of IgG signal. Exposure to TSA did result in the observation of p50 binding to this region of the promoter (Figure 6.7 B).

6.7.3 The CACNA1G Calcium Channel Promoter

6.7.3.1 The CACNA1G Promoter CpG1 Region

In the un-stimulated sample SP1 CBP, MZF and RelA were observed to occupy this region of the promoter (Figure 6.9 A I and II). Following exposure to TNF, only CBP and RelA binding were observed bound to this region of the promoter (Figure 6.9 A I and II). Following exposure to TSA the promoter was seen to be bound by SP1, CBP, MZF and RelA as seen in the un-stimulated samples but in addition p50 was also observed binding to this region of the promoter. However all TSA stimulated ChIPs for SP1, CBP and MZF also displayed high intensity IgG signals.

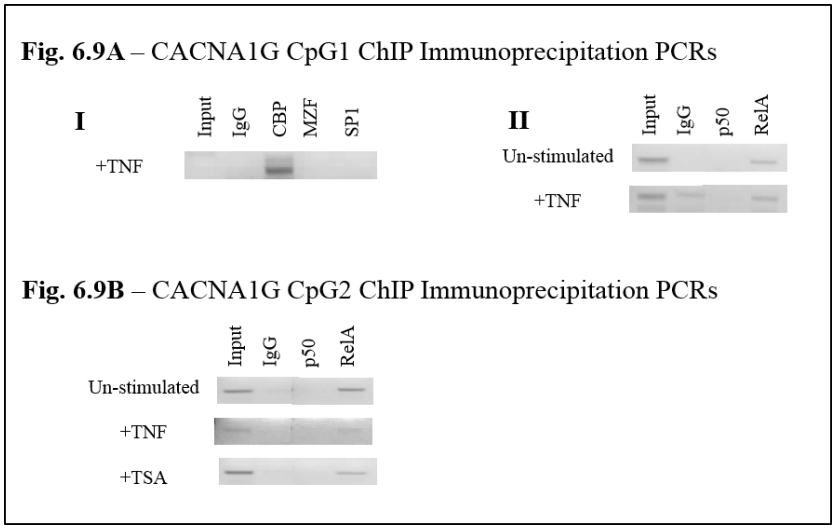


Figure 6.9 CACNA1G Promoter ChIP PCRs – Representative Gels.
ChIP chromatin was immunoprecipitated using CBP, MZF, SP1 (I), p50, RelA (II) or non-specific rabbit IgG, the resulting chromatin was then amplified using either the CACNA1C CpG1 promoter specific primers **(A)** or the CACNA1C CpG2 promoter specific primers **(B).** The IgG signal intensities varied greatly between repeats and between the different PCRs. Specific signal within the IP lane also varied greatly. No further analysis was undertaken.

6.7.3.2 The CACNA1G Promoter CpG2 Region

In the un-stimulated sample SP1 CBP, MZF were observed to occupy this region of the promoter, however, these ChIPs also displayed high intensity IgG signals. RelA was also seen to occupy this region of the promoter (Figure 6.8 B). Unlike the CACNA1G CpG1

region, exposure to either TNF or TSA had no visual effect on the observed transcription factor binding in this region (Figure 6.8 B).

6.8 Methylation of the Channel Promoters

As with the ChIP assay, the MeDIP assay PCRs were again repeatedly inconsistent, displaying a high degree of variability in the input, IgG and specific signal intensities. This made analysis difficult and any meaningful interpretation of the analysis impossible. Therefore, only simple observations from the MeDIP PCRs are discussed below.

There was some methylation observed on the 3' KCNMA1 promoter region in the un-stimulated and TNF stimulated samples (Figure 6.9). However, continual high background precluded any meaningful analysis. No methylation was observed following exposure to TSA (Figure 6.9).

Some methylation of the CACNA1C CpG 2 promoter region was evident in the un-stimulated samples. This methylation was also evident following both TNF and TSA stimulation (Figure 6.9)

Some methylation of the CACNA1G CpG 2 promoter region was evident in the un-stimulated samples. This methylation was also evident following both TNF and TSA stimulation (Figure 6.9)

Fig. 6.10– MeDIP Immunoprecipitation PCRs - Representative Gel

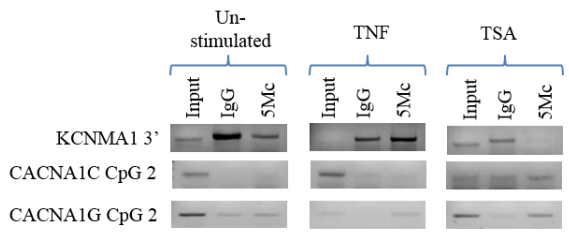


Figure 6.10 KCNMA1, CACNA1C and CACNA1G Promoter MeDIP PCRs
MeDIP chromatin was immunoprecipitated using 5-Methyl Cytosine antisera or non-specific rabbit IgG, the resulting chromatin was then amplified using the channel promoter specific primers. The IgG signal intensities varied greatly between repeats and between the different PCRs. Specific signal within the IP lane also varied greatly. No further analysis was undertaken.

6.10 Discussion

There were confounding factors when it came to interpreting the results of the ChIP and MeDIP assays. Firstly, the promoter regions of the three channels were GC-rich making PCR optimisation difficult. Regions of DNA with high GC content are more stable than regions with low GC content. These regions have a tendency to form complex secondary structures meaning that they are more resistant to melting. In addition to this, the primers designed for GC-rich regions are more likely to form self- or cross-dimers and can also form stem-loop structures which then impedes the progress of the DNA polymerase. This means that there can be a high proportion of incomplete DNA templates formed during the PCR reaction. To overcome these problems, DMSO was used in the PCR reactions as it disrupts the base pairing, reducing secondary structure and making the DNA more labile for heat denaturation, DMSO also facilitates primer annealing and hence improves amplification.

Secondly, even after optimisation of the PCR, the results were repeatedly inconsistent, with the same reaction mix and times producing clear results in one experiment and poor results in a second experiment. GC-rich regions are not only more stable they are also less specific, meaning they are more prone to mis-priming with other GC-rich regions; this leads to inconsistent results. The only option to overcome this was to re-design the primers so they were less GC-rich. This, however, was not an option due to the constraints of the ChIP assay. ChIP primers have to be designed within the promoter region in order to be able to interpret the results effectively, and the promoter regions in question are GC-rich.

Finally, the myometrial cell cultures were not cell cycle synchronised prior to the ChIP assay. The principle behind cell synchronisation is to ensure all the cells in culture are at the same phase in the cell cycle; the assumption is then made that synchronised cells will all respond identically to stimuli. The decision was taken not to synchronise the cells for the following reason. Progression through the cell cycle has been shown to be regulated by both potassium and calcium channels. Potassium channels are responsible for hyperpolarising the plasma membrane, which is necessary for the cells to move from G_1 to S (Wonderlin and Strobl 1996, Ouadid-Ahidouch and Ahidouch 2013). Calcium

channels are thought to provide transient signals at checkpoints within the cell cycle which are necessary for the cell to continue cycling (Whitaker 2006).

Serum starvation which is routinely used to synchronise cells has been shown to have an effect on the expression of the KCNMA1, CACNA1C and CACNA1G channels. It causes an up regulation of both the KCNMA1 and CACNA1C and a down regulation of the CACNA1G (Woodfork, Wonderlin *et al.* 1995, Panner, Cribbs *et al.* 2005, Patel, Clunn *et al.* 2005). These changes in regulation of transcription of the channels may obscure changes in regulation due to TNF and TSA. Instead of synchronising the cells the decision was taken to grow the cell cultures to complete confluence as within vascular tissue, smooth muscle cells have been shown to switch from a proliferative to a contractile phenotype (Charron, Nili *et al.* 2006) upon reaching confluence. This has primarily been studied in vascular smooth muscle cells and in these cells this switch in phenotype is accompanied by changes in the expression of both potassium and calcium channels. By growing the cells until they were confluent in the culture flasks the majority of cells should have moved out of the proliferative phenotype into the contractile phenotype and hence any changes in transcriptional regulation would be more reflective of this contractile phenotype and not due to the cells being in different phases of the cell cycle.

6.11 Conclusion

Unfortunately due to the difficulties encountered while carrying out the ChIP experiments on the channel promoters, it has not been possible to reach any conclusion regarding the regulation of the transcription of any of the channels. Further work is needed to circumvent these issues in order to be able to generate consistent results which can then be analysed and interpreted. It was decided that the focus should now switch to the examining putative role of the KCNMA1 channel within the nucleus. The transcription factor CCAT has been shown to be generated from the C-terminal tail of the CACNA1C channel protein (Gomez-Ospina, Tsuruta *et al.* 2006), therefore, the nuclear localisation of KCNMA1 observed in Chapter 3 Figure 3.1 D I and Chapter 4 Figure 4.10 may indicate that the C-terminal of KCNMA1 channel may also function as a transcription factor.

Chapter 7: ChIP Sequencing

7.1 Introduction

Immunocytochemistry staining of the primary myocyte cultures and western blotting of both the soluble nuclear and chromatin bound protein fractions revealed nuclear localisation of the KCNMA1 channel. In order to determine if this nuclear-localised KCNMA1 protein bound to DNA and hence had a role in the regulation of transcription it was decided to undertake a proof of concept study by utilising the ChIP assay followed by DNA sequencing (ChIP-seq). This approach was decided upon as there was no information regarding what genes the KCNMA1 protein may be associated with and therefore there were no known targets for standard PCR analysis of the ChIP DNA. The advantage of using ChIP-seq as opposed to the conventional PCR or ChIP-on-chip approaches is that ChIP-seq sequences all the DNA found to be associated with the KCNMA1 protein, these sequences can then be aligned with the genome and subsequently with individual genes. In this way it is possible to reveal the full complement of KCNMA1 protein:DNA associations within the nucleus. ChIP-seq also gives improved signal-to-noise ratio, is able to detect more peaks and the detected peaks are narrower in comparison ChIP-on-chip (Ho, Bishop *et al.* 2011).

This putative dual role of the KCNMA1 protein as an ion channel and a transcription factor, although novel for the KCNMA1 protein, has been reported previously for the CACNA1C protein. In this channel the C-terminus has been shown to enter the nucleus and regulate gene transcription (Gomez-Ospina, Tsuruta *et al.* 2006).

The CACNA1C transcription factor, termed CCAT, is generated from the C-terminal tail of the CACNA1C calcium channel protein and its entry into the nucleus is negatively regulated by the electrical activity of the cell. CCAT can also bind to the remainder of the channel protein reducing calcium influx through the channel. In this way it is similar to the potassium channel binding protein Kchip/DREAM. These proteins interact with Kv K^+ channels increasing potassium efflux (Bähring, Dannenberg *et al.* 2001) and also act as transcriptional repressors (Venn, Haynes *et al.* 2008). The production of the CCAT fragment was not found to be a regulated process. However its nuclear localisation and subsequent transcriptional activity was found to be regulated by intracellular calcium levels; with a reduction in intracellular calcium promoting nuclear localisation and an increase repressing nuclear localisation. Once within the nucleus, CCAT was found to

up-regulate a number of genes including gap junction membrane channel protein beta 5 and EGR-1, it also down-regulated a number of genes such as the transcription factor GATA6 and the potassium intermediate/small conductance calcium activated channel Kcnn3 (Gomez-Ospina, Tsuruta *et al.* 2006). Further research has demonstrated that the CCAT transcription factor is generated from a promoter within the coding region of the CACNA1C gene (Gomez-Ospina, Panagiotakos *et al.* 2013). This internal promoter drives the expression of exons 46 and 47 independently, however, they also demonstrated that CCAT and the CACNA1C protein are expressed in a complementary fashion in the developing brain this would suggest that there is still a link between the expression of these two proteins. The CCAT fragment is approximately 75kDa in size, the size of the KCNMA1 protein localised to the nucleus is between approximately 65kDa and 138kDa, the smaller of these is comparable with CCAT.

As explained above ChIP-seq is a powerful tool used to map global DNA binding sites for any protein of interest. The workflow for ChIP-seq firstly involves characterising the antibody to be used, then using the ChIP assay to selectively enrich for DNA sequences bound to a particular protein. Oligonucleotide adaptors are then added to the fragments of DNA isolated in the ChIP assay to enable these fragments to be sequenced using parallel sequencing. Finally, the ChIP fragments are sequenced, this involves size selection for fragments that are approximately 300bp long. These fragments are then sequenced simultaneously using a genome sequencer. In this study, cluster amplification was utilised, this involves creating clusters of approximately 1000 clonal copies of each of the adapter ligated ChIP DNA fragments on a solid flow cell substrate. This then produces a high density array of clusters on the flow cell surface which is then sequenced by a genome analysing program. Each cluster is sequenced in parallel base by base during each read using novel fluorescently labelled reversible terminator nucleotides. This data is then collect as .fastq files which can then be aligned to the genome and individual genes identified.

Characterisation of the antibody to be used is important as the quality of a ChIP experiment and hence the downstream ChIP-seq is governed by the specificity of the antibody and the degree of enrichment achieved in the immunoprecipitation. The ENCODE and modENCODE consortia recommend using a primary and secondary test to characterise each monoclonal antibody (Landt, Marinov *et al.* 2012). The primary tests

recommended are either a western blot or immunofluorescence staining. These will provide information on nuclear localisation, cross-reactivity and any isoforms detected by the antibody. The secondary tests are then, factor "knockdown", ChIP using antibodies against a different epitope or a different member of a complex, immunoprecipitation using epitope tagged constructs or binding site motif analysis. These tests ensure the antibody is suitable for use in the ChIP assay and add confidence to the data obtained from ChIP-seq.

The sensitivity of the ChIP-seq is dependent on a number of factors including, the depth of the sequencing run, the library complexity and the distribution of the target protein. The depth of the sequencing run is the number of mapped sequence tags, for example a 'lane' can generate 200 million reads and so if 20 libraries are loaded it can generate 10million reads per library. The ENCODE and modENCODE consortia recommend a minimum of 20million mapped reads per sample in order to have confidence in the data (Landt, Marinov *et al.* 2012). Library complexity, the amount of unique DNA molecules in the sample also affects the sensitivity of ChIP-seq, this can be affected by both the genome being studied and the amount of DNA recovered from the ChIP assay. Finally the distribution of the target protein; this will depend on what the protein of interest is. Transcription factors tend to be "point source" factors which produce localised sharp peaks, these are easier detected and as a result the ChIP-seq is more sensitive (Bailey, Krajewski *et al.* 2013).

Once the .fastq files are generated it is then necessary to utilise NGS analysis software to align the reads to the genome, perform peak calling to identify regions of ChIP enrichment, and if possible to identify DNA binding site motifs. In this study Galaxy (https://usegalaxy.org) an open access web based platform was used for NGS analysis (Giardine, Riemer *et al.* 2005, Blankenberg D, Von Kuster G *et al.* 2010, Goecks, Nekrutenko *et al.* 2010). Within this platform Bowtie for Illumina was used to align the reads to the genome, this uses a Burrows-Wheeler transformation to create a permanent, reusable index of the genome. Greater than 80% of the reads should be mapped otherwise this indicates that there is an issue with the sequencing data. Peak calling uses algorithms to identify regions which have more reads than the background in this study the SCIER algorithm was used (Zang, Schones *et al.* 2009). Finally, motif identification was

undertaken, this was done using only the highest confidence peaks and the Panoptic Motif Search Tool (Dinh and Rajasekaran 2013).

7.2 Antibody Characterisation

As this was a proof-of-concept study it was decided that it would be best use one of the two antibodies which had been used previously during immunocytochemistry and Western Blotting. The ENCODE and modENCODE consortia recommend utilising a two stage antibody characterisation were the primary test can be a western blot, as these two antibodies have been used in western blots already this was utilised as the primary test. The C-terminal antibody gave much cleaner western blots which revealed nuclear localisation (Chapter 4 Figures 4.5 and 4.9A), no apparent cross reactivity and only two isoforms of the KCNMA1 protein (138kDa and 65kDa) and therefore this antibody was selected. It is not possible at this stage to determine conclusively if either the 138kDa or the 65kDa KCNMA1C proteins are used to generate this putative transcription factor. However, the protein detected within the chromatin bound nuclear fraction in chapter 4 Figure 1.10B was larger than 65kDa so it is more probable that it is generated from the 138kDa KCNMA1 protein. A second possibility is that, like CCAT, the putative transcription factor is transcribed from an internal promoter. No secondary test was undertaken at this stage but this will need to be done prior to any further experimentation should the KCNMA1 protein be shown to act as a transcription factor.

7.3 The ChIP Assay

In order to study the putative transcription factor activity of the KCNMA1 channel the ChIP assay was used. Briefly, six T-75 flasks of primary myocytes were grown to 100% confluence these were left unstimulated. The ChIP assay was carried out as detailed in chapter 2 *Material and Methods* Section 2.7. The samples included three repeats each of input and KCNMA1 immuoprecipitated chromatin. It was not possible to carry out a control PCR to determine if the ChIP had been successful - as stated previously this is a novel putative transcription factor and therefore there is no known target to serve as a positive control. After quantification of the ChIP chromatin it was apparent that there was insufficient chromatin for use in the sequencing reaction (Table 7.1). To circumvent this issue whole genome amplification was carried out, as detailed in chapter 2: *Materials and*

Methods Section 2.10.1.1, on the ChIP chromatin this gave sufficient DNA for ChIP sequencing (Table 7.2).

Table 7.1 Quantification of ChIP Chromatin

Sample	Input (total)	KCNMA1 IP (total)
Rpt 1	4.0ng/ul (180ng)	4.5ng/ul (202ng)
Rpt 2	4.5ng/ul (202ng)	2.0 ng/ul (90ng)
Rpt 3	1.0ng/ul (45ng)	2.0 ng/ul (90ng)

A portion of the chromatin was analysed by 2% AGE in order to determine if the fragments were in the correct size range (100-500bp; Figure 7.1). Figure 7.1 shows the majority chromatin fragments are between 100 – 200bp with the smear extending up to a maximum of 500bp.

Table 7.2 Quantification of ChIP Chromatin Following Whole Genome Amplification

Sample	A260/A280	A260/A230	ng/µl	Total ng in 25µl
Input R1	1.793	2.364	130	3,250
KCNMA1 IP R1	1.607	1.216	113	2,825
Input R2	1.833	2.276	165	4,125
KCNMA1 R2	1.683	2.156	173	4,325
Input R3	1.724	2.174	125	3,125
KCNMA1 R3	1.811	2.030	168	4,200

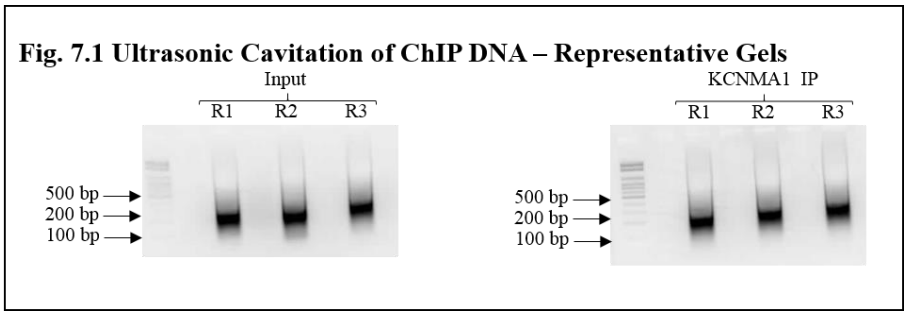


Figure 7.1 Ultrasonic Cavitation of ChIP DNA - Representative Gels
Formaldehyde fixed nuclei isolated during the ChIP assay were sonicated on ice for 3 x 10 second intervals. The sheared DNA was visualised on a 1.5% (w/v) agarose gel and calibrated against a LowRanger ladder (M). The DNA shears were most intense between 200 and 100bp with a smear extending up to 500bp.

7.4 ChIP Sequencing

ChIP sequencing was conducted by Dr Paul Heath, SITraN. The first step in the process was library preparation, this was initially undertaken using the Kapa Biosystems Library preparation Kit (Kapa Biosystems KR0426), however, after starting it was discovered that the required adapters were not included in the kit. It was therefore necessary to switch to the NEB Next Ultra DNA library preparation kit Multiplex Oligos for Illumina (Index

Primers Set 1; New England Biolabs E7370) and the library preparation was completed with this kit. Although the ChIP samples have a range of fragment sizes, this library preparation preferentially selects fragments of ~300bp and in this way, the library loaded onto the flow cells consists of approximately equal fragment sizes. All the prepared libraries where then loaded onto lane five of the flow cells at a concentration of 10pM and onto lane six of the flow cell at a concentration of 8pM. The library samples were prepared and then sequenced as paired-end reads. This means that the sequence is read from each end of the molecule. Paired-end reads, in addition to knowing the length of the library fragments, gives one sufficient information to then map the reads to a reference genome. Table 7.3 details the labels for the samples on the Illumina HiScan SQ system and how these relate to the ChIP samples (Table 7.3).

Table 7. 3 Sample Labels for ChIP-seq

Sample	ChIPseq Label	Information
Input Chromatin Repeat 1	Rpt1-input CGATGT L005 R1 001	Paired end reads loaded on lane 5
	Rpt1-input CGATGT L005 R2 001	
	Rpt1-input CGATGT L006 R1 001	Paired end reads loaded on lane 6
	Rpt1-input CGATGT L006 R2 001	
KCNMA1 IP Chromatin Repeat 1	Rpt1-KCNMA1 TGACCA L005 R1 001	Paired end reads loaded on lane 5
	Rpt1-KCNMA1 TGACCA L005 R2 001	
	Rpt1-KCNMA1 TGACCA L006 R1 001	Paired end reads loaded on lane 6
	Rpt1-KCNMA1 TGACCA L006 R2 001	
Input Chromatin Repeat 2	Rpt2-input ACAGTG L005 R1 001	Paired end reads loaded on lane 5
	Rpt2-input ACAGTG L005 R2 001	
	Rpt2-input ACAGTG L006 R1 001	Paired end reads loaded on lane 6
	Rpt2-input ACAGTG L006 R2 001	
KCNMA1 IP Chromatin Repeat 2	Rpt2-KCNMA1 GCCAAT L005 R1 001	Paired end reads loaded on lane 5
	Rpt2-KCNMA1 GCCAAT L005 R2 001	
	Rpt2-KCNMA1 GCCAAT L006 R1 001	Paired end reads loaded on lane 6
	Rpt2-KCNMA1 GCCAAT L006 R2 001	
Input Chromatin Repeat 3	Rpt3-input CAGATC L005 R1 001	Paired end reads loaded on lane 5
	Rpt3-input CAGATC L005 R2 001	
	Rpt3-input CAGATC L006 R1 001	Paired end reads loaded on lane 6
	Rpt3-input CAGATC L006 R2 001	
KCNMA1 IP Chromatin Repeat 3	Rpt3-KCNMA1 CTTGTA L005 R1 001	Paired end reads loaded on lane 5
	Rpt3-KCNMA1 CTTGTA L005 R2 001	
	Rpt3-KCNMA1 CTTGTA L006 R1 001	Paired end reads loaded on lane 6
	Rpt3-input CTTGTA L006 R2 001	

7.5 Sequencing Run Quality Control

A number of quality controls are undertaken during the sequencing run, the first of these is analysis of the raw cluster counts. The raw cluster counts should have a maximum of 950,000, on the graph in Figure 7.2 blue is the raw count data and green is the percentage

that have passed the quality scoring. The Q30 value for the green bars should be greater than 80%, for this sequencing run the Q30 values were 75.1 and 76.4% these are a little lower than the recommended value (Figure 7.2 A, B).

Fig. 7.2A Raw Cluster Counts for Flow Cell Lane Five

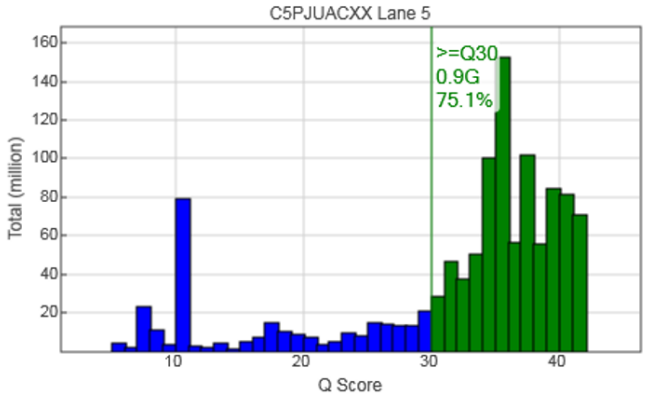


Fig. 7.2B Raw Cluster Counts for Flow Cell Lane Six

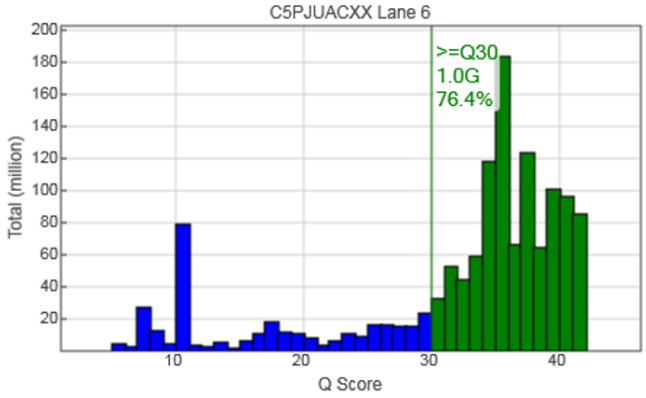


Figure 7.2 Raw Cluster Counts
ChIP fragments were sequenced simultaneously on a genome sequencer by cluster amplification. Clusters of approximately 1000 clonal copies of each of the adapter ligated ChIP DNA fragments are created on a solid flow cell substrate. Producing a high density array of clusters on the flow cell surface which is then sequenced by a genome analysing program. At cycle four of the sequencing reaction a count of the raw clusters is performed. The bars represent the raw count data and the blue bars are those that have passed quality scoring**. (A)** Raw cluster counts for flow cell lane 5. 75.1% of clusters passed the quality control check**. (B)** Raw cluster counts for flow cell lane 6. 76.4% of clusters passed the quality control check

Next, at cycle four, the cluster density for each lane is plotted as a box plot. The samples for this study were loaded in lanes five and six, in figure 7.3 it is evident that that the cluster density in these lanes is much lower than in the other lanes (Figure 7.3). Later in the run, however, the cluster density improved and these lanes were equivalent to the other lanes. At cycle 12, the phasing and pre-phasing data is checked. Phasing occurs when sequencing of some molecules in the in the cluster falls behind by one or more

cycles. Pre-phasing occurs when sequencing of some molecules in the in the cluster skips ahead by one or more cycles. Therefore, the percentage phasing/pre-phasing is a measure of the number of molecules in a cluster which have either fallen behind or skipped ahead. Libraries with low diversity or unbalanced libraries can have high levels of phasing/pre-phasing. The percentage phasing/pre-phasing should be less than 0.5%, this was the case in lanes five and six (Table 7.4). At the 25th cycle the chastity filter results are displayed. Each base gives off a signal and the base with the higest intensity signal is the base called. The chastity filter measures the purity of the signal from each cluster and removes clusters that have too much intensity corresponding to bases other than the called base. It is calculated by dividing the higest intensity signal from the called base and dividing this by the higest intensity from the called base plus the next higest intensity signal. The purpose of the chastity filter is to remove data which does not meet the overall quality criteria of the filter. The results are displayed as Clusters PF % (passing filter) in the analysis report within lanes five and six less than 50% of the clusters passed the filter (Table 7.4). This is low and indicates that either the ChIP assay or the library preparation or both need optimising.

Fig. 7.3 Cluster Density as Calculated at Cycle Four

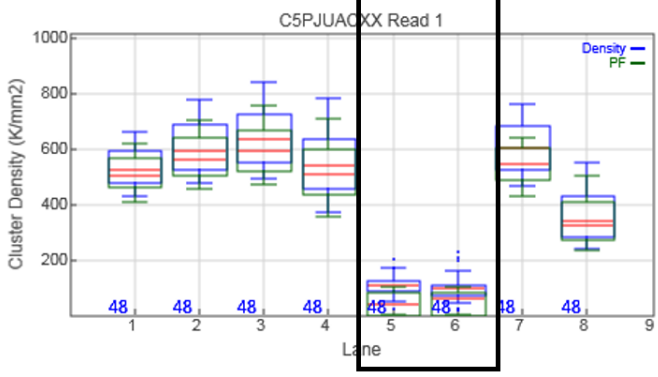


Figure 7.3 Cluster Density
ChIP fragments were sequenced simultaneously on a genome sequencer by cluster amplification. Clusters of approximately 1000 clonal copies of each of the adapter ligated ChIP DNA fragments are created on a solid flow cell substrate. Producing a high density array of clusters on the flow cell surface which is then sequenced by a genome analysing program. At cycle four of the sequencing reaction a measure of the cluster density is performed. The box plots represent the cluster density, flow cell lane 5 and 6 box plots are highlighted with a black box. The cluster density for both flow cell lanes 5 and 6 are low.

Table 7.4 ChIP-seq run Quality Control Checks

Lane	Clusters PF (%)	Phas/Prephas (%)	Clusters PF (%)	Phas/Prephas (%)	Clusters PF (%)	Phas/Prephas (%)	Clusters PF (%)	Phas/Prephas (%)
5	38.98 +/- 36.72	0.155 / 0.294	38.98 +/- 36.72	0.000 / 0.000	38.98 +/- 36.72	0.000 / 0.000	38.98 +/- 36.72	0.011 / 0.018
6	49.45 +/- 35.41	0.212 / 0.403	49.45 +/- 35.41	0.000 / 0.000	49.45 +/- 35.41	0.000 / 0.000	49.45 +/- 35.41	0.005 / 0.007

7.6 Quality Control of Data on Galaxy

For quality control purposes summary statistics was computed for each of the .fastq files generated by the sequencing. The FastQC quality control gives information regarding the quality of the .fastq data. For the following quality controls data is only shown for the paired end reads from both input and KCNMA1 immunoprecipitated chromatin repeat one loaded in flow cell lane five.

7.6.1 Basic Statistics

This gives simple composition statistics for the data set, including total number of sequences, sequence length and percentage GC. Table 7.5 shows that these basic statistics are consistent across all the data sets.

7.6.2 Per Base Sequence Quality

This plot represents the range of quality values over all the bases at each position, interestingly the reverse read of the paired end reads (R2) for each sample in each lane failed this quality assessment but the forward read of the paired end reads (R1) all passed (Figure 7.4 A, B).

Table 7.5 ChIP-seq Basic Statistics

	No of Sequences	Poor Quality Seq	Sequence Length	%GC
Rpt1 Input L005 R1	873205	0	93	43
Rpt1 Input L005 R2	873205	0	93	43
Rpt1 Input L006 R1	1016194	0	93	43
Rpt1 Input L006 R2	873205	0	93	43
Rpt1 KCNMA1 L005 R1	1012337	0	93	44
Rpt1 KCNMA1 L005 R2	1012337	0	93	43
Rpt1 KCNMA1 L006 R1	1159413	0	93	44
Rpt1 KCNMA1 L006 R2	1159413	0	93	43
Rpt2 Input L005 R1	752076	0	93	43
Rpt2 Input L005 R2	752076	0	93	43
Rpt2 Input L006 R1	873673	0	93	43
Rpt2 Input L006 R2	873673	0	93	43
Rpt2 KCNMA1 L005 R1	1164338	0	93	43
Rpt2 KCNMA1 L005 R2	1164338	0	93	43
Rpt2 KCNMA1 L006 R1	1353323	0	93	43
Rpt2 KCNMA1 L006 R2	1353353	0	93	43
Rpt3 Input L005 R1	833431	0	93	43
Rpt3 Input L005 R2	833431	0	93	43
Rpt3 Input L006 R1	989943	0	93	43
Rpt3 Input L006 R2	989943	0	93	43
Rpt3 KCNMA1 L005 R1	950257	0	93	44
Rpt3 KCNMA1 L005 R2	950257	0	93	43
Rpt3 KCNMA1 L006 R1	1115135	0	93	44
Rpt3 KCNMA1 L006 R2	1115135	0	93	43

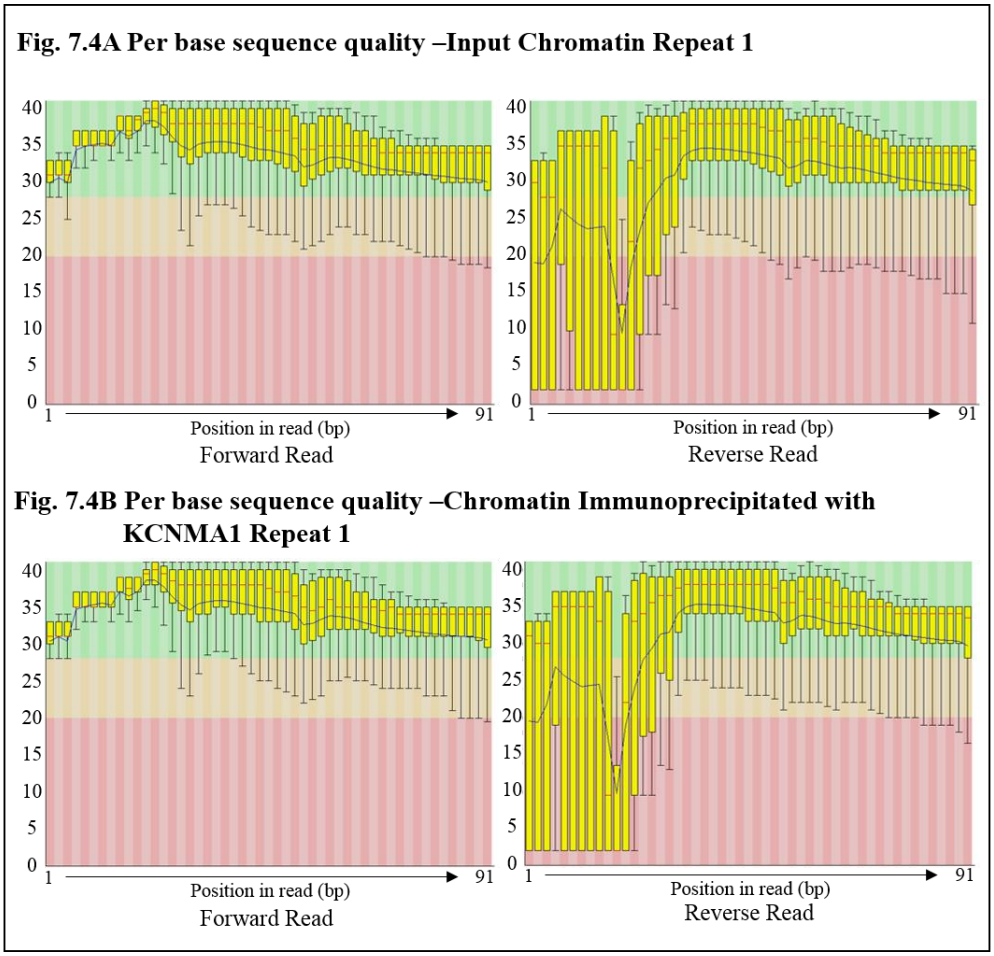


Figure 7.4 Per Base Sequence Quality
A measure of the probability that a base is called incorrectly, the Phred Score, was calculated for each base in the sequence. For each position a BoxWhisker plot was drawn. The central red line is the median value, the yellow box represents the inter-quartile range (25-75%), the upper and lower whiskers represent the 10% and 90% points and the blue line represents the mean quality. The y-axis shows the quality scores. The background of the graph is divided into very good quality reads (green), reasonable quality reads (orange) and poor quality reads (red). (A) Per base sequence quality for Input repeat 1. The forward read passed this quality control check (left panel), the reverse read failed this check as the lower quartile for a number of bases was less than 5 and the median for a number of bases was less than 20 (right panel). (B) Per base sequence quality for KCNMA1 IP repeat 1. The forward read passed this quality control check (left panel), the reverse read failed this check as the lower quartile for a number of bases was less than 5 and the median for a number of bases was less than 20 (right panel).

7.6.3 Per Sequence Quality Scores

This quality control identifies if there is a subset of the sequences which have low scores. A good quality dataset should have a mean quality score of 37, whilst a poor quality data set will have a mean quality score of around 17. These values are determined using the Phred algorithm, which is a measure of the probability that a base is called incorrectly. For example a Phred score of 30 means that there is a 1:1000 probability of an incorrect base call giving a base call accuracy of 99.9%. All of the sample reads passed this test with a peak at around 37, a number of the samples also had a peak of around 29 (Figure

7.5 A, B). This should not be a problem as it routine to count bases with a quality score of greater than 20.

Fig. 7.5A Per sequence quality score –Input Chromatin Repeat 1

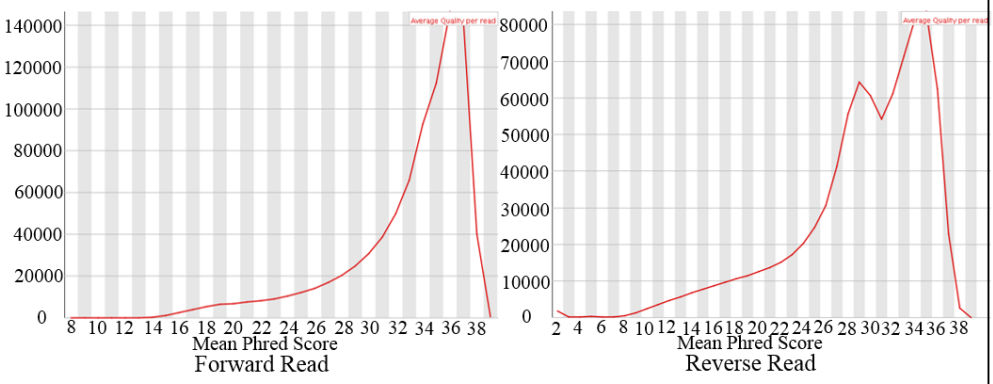


Fig. 7.5B Per sequence quality Score –Chromatin Immunoprecipitated with KCNMA1 Repeat 1

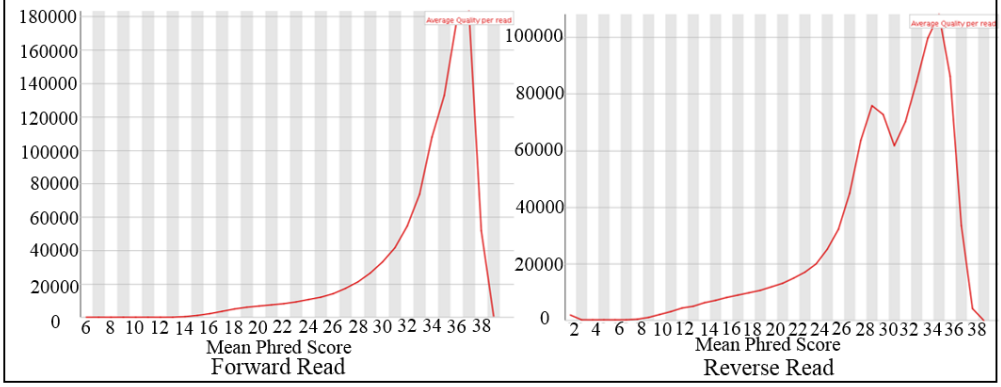


Figure 7.5 Per Sequence Quality Score
A measure of the probability that a base is called incorrectly – The Phred Score, was calculated for each sequence. This quality control identifies if there is a subset of the sequences which have low scores. A good dataset should have a mean quality score of 37, whilst a bad data set will have a mean quality score of around 17. The x-axis shows the quality scores. (A) Per sequence quality score for Input repeat 1. The forward read passed this quality control check (left panel), the reverse read also passed this check (right panel). (B) Per sequence quality score for KCNMA1 IP repeat 1. The forward read passed this quality control check (left panel), the reverse read also passed this check (right panel).

7.6.4 Per Base Sequence Content

This shows the proportion of each base at each position in the read. In a random library the base frequency should be approximately equal at all positions over all the reads. If there are strong biases which change for different bases this represents an overrepresented sequence which is contaminating the library. All datasets failed this quality control, this appears to be an issue with the first 30bp of each sequence as beyond this point they have

passed the quality control check (Figure7.6 A, B). This may be associated with either the adapters or the whole genome amplification step that was undertaken.

Fig. 7.6A Per base sequence content –Input Chromatin Repeat 1

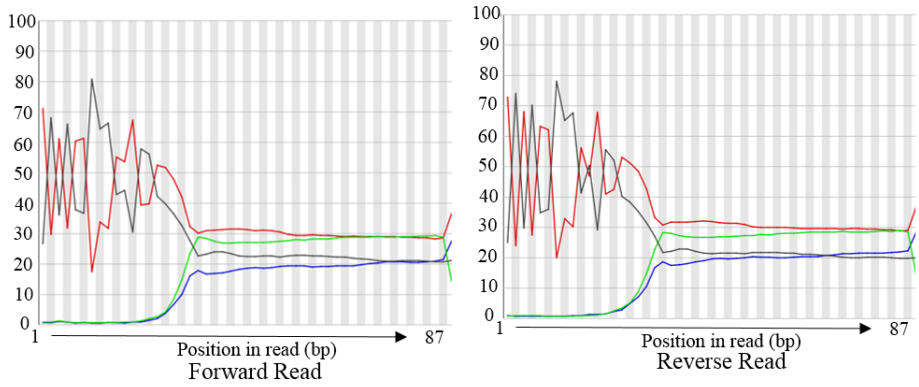


Fig. 7.6B Per base sequence Content – Chromatin Immunoprecipitated with KCNMA1 Repeat 1

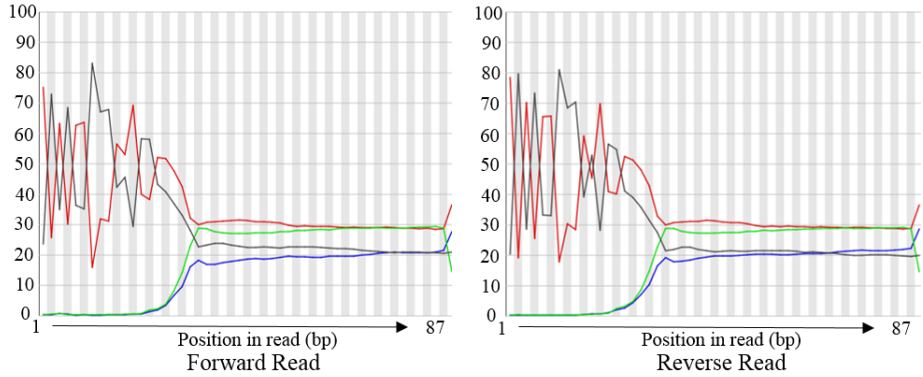


Figure 7.6 Per Base Sequence Content
Plot of the proportion of each base position in a file for which each of the four normal DNA bases has been called. The x-axis shows the position in the read and the y-axis shows the percentage. In a random library all four lines should run roughly parallel to each other. (A) Per Base Sequence Content for Input repeat 1. Both the forward (left panel) and reverse (right panel) failed this quality control check as the difference between A and T, and G and C were greater than 20% in a number of positions. (B) Per Base Sequence Content for KCNMA1 IP repeat 1. Both the forward (left panel) and reverse (right panel) failed this quality control check as the difference between A and T, and G and C were greater than 20% in a number of positions.

7.6.5 Per Sequence GC Content

This plot show the GC content across each sequence. A random library there should be little difference between positions and the overall GC content should be similar to that seen in the genome. Deviations from this can indicate that there is an over-represented sequence, or there has been a problem in the library preparation. All but one of the data sets passed this quality control (Figure 7.7 A, B); the one exception was Rpt3 KCNMA1

L006 R2 this didn't fail the quality control but had a caution as the distribution was slightly offset from normal (Data not shown).

Fig. 7.7A Per sequence GC content–Input Chromatin Repeat 1

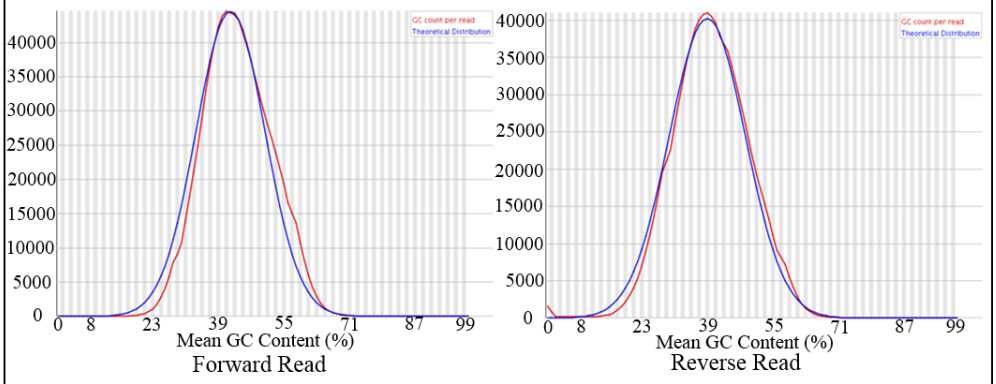


Fig. 7.7B Per sequence GC content – Chromatin Immunoprecipitated with KCNMA1 Repeat 1

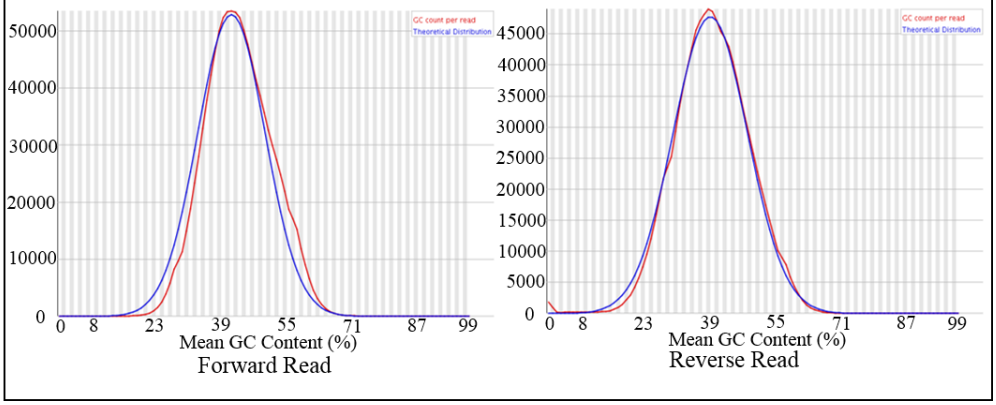


Figure 7.7 Per Sequence GC Content
This measures the GC content across the whole length of the sequence (Red line) and compares it to a modelled normal distribution of GC content (Blue line). The x-axis shows the mean GC content. (A) Per sequence GC content for Input repeat 1. The forward read passed this quality control check (left panel), the reverse read also passed this check (right panel). (B) Per sequence GC content for KCNMA1 IP repeat 1. The forward read passed this quality control check (left panel), the reverse read also passed this check (right panel).

7.6.6 Duplicate Sequences

This counts the number of times any sequence occurs in the data set and then plots the relative number of sequences with different degrees of duplication. Diverse libraries should not have a high degree of duplication, low level of duplication may indicate a high level of coverage of the target sequence. High levels of duplication are indicative of some kind of enrichment bias. All the samples passed this quality control as the percentage of duplicated sequences was below 0.72% (Table 7.6, Duplicate Sequences).

7.6.7 Over-represented Sequences

This creates a list of all the sequences in the library that constitute over 0.1% of the total library. Finding an over-represented sequence can mean either it is highly biologically significant or the library is contaminated. Interestingly the R2 reads for each sample in each lane had a caution for this quality assessment due to the high number of N bases but the R1 reads all passed (Table 7.6, Overrepresented Sequences). N bases are called during sequencing when the sequencer detects either missing peaks or where there are multiple peaks for different nucleotides at a single position. Therefore the presence of a large number of N calls in the reverse reads may indicate a problem with the chromatin or the reverse sequencing is of a poor quality. The R2 reads did not fail this quality check, as no sequences were found to represent more than 1% of the total.

Table 7.6 ChIP-seq Quality Control Checks

	Duplications	Overrepresented Sequence
Rpt1 Input L005 R1	0.16	Pass
Rpt1 Input L005 R2	0.27	Caution
Rpt1 Input L006 R1	0.35	Pass
Rpt1 Input L006 R2	0.27	Caution
Rpt1 KCNMA1 L005 R1	0.13	Pass
Rpt1 KCNMA1 L005 R2	0.25	Caution
Rpt1 KCNMA1 L006 R1	0.3	Pass
Rpt1 KCNMA1 L006 R2	0.6	Caution
Rpt2 Input L005 R1	0.14	Pass
Rpt2 Input L005 R2	0.28	Caution
Rpt2 Input L006 R1	0.31	Pass
Rpt2 Input L006 R2	0.65	Caution
Rpt2 KCNMA1 L005 R1	0.2	Pass
Rpt2 KCNMA1 L005 R2	0.31	Caution
Rpt2 KCNMA1 L006 R1	0.3	Pass
Rpt2 KCNMA1 L006 R2	0.64	Caution
Rpt3 Input L005 R1	0.09	Pass
Rpt3 Input L005 R2	0.26	Caution
Rpt3 Input L006 R1	0.21	Pass
Rpt3 Input L006 R2	0.66	Caution
Rpt3 KCNMA1 L005 R1	0.2	Pass
Rpt3 KCNMA1 L005 R2	0.37	Caution
Rpt3 KCNMA1 L006 R1	0.33	Pass
Rpt3 KCNMA1 L006 R2	0.72	Caution

7.8 Data Analysis on Galaxy

The first step in the data analysis was to map the reads to a reference genome, the human hg19 reference genome was used for this. Unfortunately it was only possible to map one repeat of each of the input and KCNMA1 immunoprecipitation .fastq files this was the

input and KCNMA1 immunoprecipitated chromatin repeat one loaded in flow cell lane five. After mapping the data, peak calling was performed using SICER comparing input to immunoprecipitation. Peak calling is used to identify regions of the genome that are enriched in the immunoprecipitated samples in comparison to the input controls, these represent areas where the protein of interest, KCNMA1 C-terminal domain, binds to the DNA. SICER is a statistical clustering based approach utilised to perform peak calling. This resulted in 48 regions which were significantly enriched in KCNMA1 C-terminal domain binding compared to the input control. (Table 7.7).

Table 7.7 Peak Calling using SICER

Chromosome	Peak Start Position	Peak End Position	Read Counts KCNMA1	Read Counts Input	P-value	Fold Change	SICER Score
chr1	1772600	1772800	2	0	<0.05	238626.41	2.69E-16
chr1	50625000	50625200	2	0	<0.05	238626.41	2.69E-16
chr1	146897000	146897200	2	0	<0.05	238626.41	2.69E-16
chr1	187521400	187521600	2	0	<0.05	238626.41	2.69E-16
chr1	216311400	216311600	2	0	<0.05	238626.41	2.69E-16
chr2	191288200	191288400	2	0	<0.05	238626.41	2.69E-16
chr2	222789400	222789600	2	0	<0.05	238626.41	2.69E-16
chr2	239959600	239959800	2	0	<0.05	238626.41	2.69E-16
chr4	8748200	8748600	2	0	<0.05	119313.21	9.08E-16
chr4	48269400	48269600	2	0	<0.05	238626.41	2.69E-16
chr4	74360800	74361200	2	0	<0.05	119313.21	9.08E-16
chr4	97284600	97284800	2	0	<0.05	238626.41	2.69E-16
chr4	105034600	105035000	2	0	<0.05	119313.21	9.08E-16
chr4	117748800	117749200	2	0	<0.05	119313.21	9.08E-16
chr5	35748200	35748400	2	0	<0.05	238626.41	2.69E-16
chr5	115940000	115940200	2	0	<0.05	238626.41	2.69E-16
chr5	132020800	132021000	2	0	<0.05	238626.41	2.69E-16
chr5	148650400	148650600	2	0	<0.05	238626.41	2.69E-16
chr6	90557600	90557800	2	0	<0.05	238626.41	2.69E-16
chr7	121872800	121873000	2	0	<0.05	238626.41	2.69E-16
chr7	138381200	138381400	2	0	<0.05	238626.41	2.69E-16
chr8	84320000	84320200	2	0	<0.05	238626.41	2.69E-16
chr8	86322400	86322600	2	0	<0.05	238626.41	2.69E-16
chr8	106949400	106949800	2	0	<0.05	119313.21	9.08E-16
chr8	118205600	118206000	2	0	<0.05	119313.21	9.08E-16
chr8	140334000	140334200	2	0	<0.05	238626.41	2.69E-16
chr9	106480400	106480800	2	0	<0.05	119313.21	9.08E-16
chr10	32926000	32926400	2	0	<0.05	119313.21	9.08E-16
chr10	68222000	68222200	2	0	<0.05	238626.41	2.69E-16

Chromosome	Peak Start Position	Peak End Position	Read Counts KCNMA1	Read Counts Input	P-value	Fold Change	SICER Score
chr10	73954000	73954200	2	0	<0.05	238626.41	2.69E-16
chr10	81768600	81768800	2	0	<0.05	238626.41	2.69E-16
chr11	34038200	34038400	2	0	<0.05	238626.41	2.69E-16
chr11	71963400	71963600	2	0	<0.05	238626.41	2.69E-16
chr11	102480000	102480200	2	0	<0.05	238626.41	2.69E-16
chr11	103341600	103341800	2	0	<0.05	238626.41	2.69E-16
chr11	121300400	121300600	2	0	<0.05	238626.41	2.69E-16
chr13	48327400	48327800	2	0	<0.05	119313.21	9.08E-16
chr13	62802800	62803200	2	0	<0.05	119313.21	9.08E-16
chr14	27521600	27522000	2	0	<0.05	119313.21	9.08E-16
chr15	48778200	48778400	2	0	<0.05	238626.41	2.69E-16
chr18	76152400	76152600	2	0	<0.05	238626.41	2.69E-16
chr19	49387000	49387200	2	0	<0.05	238626.41	2.69E-16
chr20	38233400	38233800	2	0	<0.05	119313.21	9.08E-16
chr20	44123000	44123400	2	0	<0.05	119313.21	9.08E-16
chr21	31527000	31527200	2	0	<0.05	238626.41	2.69E-16
chrX	31004400	31004600	2	0	<0.05	238626.41	2.69E-16
chrX	67033200	67033600	2	0	<0.05	119313.21	9.08E-16
chrX	121928200	121928400	2	0	<0.05	238626.41	2.69E-16

7.9 Identification of Genes Associated with the Peaks

The NCBI genome browser was used to align the peak regions generated above with known genes (Pruitt, Brown *et al.* 2014). This done by entering the chromosomal locations identified above into the NCBI genome browser and then identifying genes within the region. Of the 48 peaks identified above 27 were able to be aligned with known genes (Table 7.8)

7.10 Identification of the Region of the Gene Corresponding to the Location of the Peak.

The next step was to identify which region of the aligned genes the peaks were associated with. If the KCNMA1 protein has transcription factor function it would be expected that the binding should be in the regulatory region of the gene. Although some transcription factors have been found bound in non-coding intronic regions of genes, the significance of this binding is not fully understood as yet. The Ensemble database (Cunningham, Amode *et al.* 2015) was used for this purpose, this database provides information

regarding the location of introns, exons and the regulatory regions of the genes. Briefly the gene was located in the Ensemble database and then the gene examined to identify which of these regions corresponded to the region covered by the peak.

Of the 27 peaks that were aligned with known genes, all 27 peaks occurred with the regulatory regions of the genes they were aligned with. Five occurred in regions annotated as the promoter region, five occurred in the promoter flanking region, four occurred in regions of known CTCF binding, six occurred in enhancer regions, two occurred in sites associated with transcription factor binding and two occurred in open chromatin. The remaining three crossed over annotated regions, one crossed between a promoter flanking region and an enhancer region, a second crossed between a promoter region and an enhancer region and the third crossed between a region of CTCF binding and an enhancer region (Table 7.8).

7.11 Gene Ontology Analysis of Peak Associated Genes

The Gene Ontology Consortium database was used to identify which biological process the genes identified above could be involved in (Ashburner, Ball *et al.* 2000, Carbon, Ireland *et al.* 2009). This revealed an interesting array of possible processes that the KCNMA1 protein could have a role in regulating. Including the G1/S transition in the cell cycle, apoptosis, Histone H3-K4 methylation, fertilisation, NFκB signalling and calcium ion homeostasis among others.

Table 7.8 Alignment of Peaks and Localisation of KCNMA1 C-terminal Domain to Genomic Regions

Chromosome	Peak Start Position	Peak End Position	Gene	Location
chr1	1772600	1772800	Nicotinamide adenine dinucleotide Kinase	Promoter
chr1	50625000	50625200	FAS-associated Factor 1	Promoter Flanking / Enhancer
chr1	146897000	146897200	Neuroblastoma breakpoint family member 12	CTCF Binding
chr1	216311400	216311600	Usher Syndrome 2A	CTCF Binding
chr2	191288200	191288400	Myosin 1B	Enhancer
chr2	222789400	222789600	Novel miRNA	Promoter Flanking

Chromosome	Peak Start Position	Peak End Position	Gene	Location
chr2	239959600	239959800	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 10	CTCF Binding
chr4	48269400	48269600	TEC Tyrosin protein kinase	Promoter
chr4	97284600	97284800	Sperm Tail PG-rich repeat containing 2	Open Chromatin
chr4	105034600	105035000	Tet methycytosine dioxygenase 2	Promoter
chr5	35748200	35748400	Sperm flagellar 2	Enhancer
chr5	148650400	148650600	5-Hydroxytryptamine (serotonin) receptor 4, G-protein coupled	Transcription Factor Binding
chr6	90557600	90557800	Mitogen activated protein kinase kinase kinase 7	CTCF Binding
chr7	121872800	121873000	Protein tyrosin phosphatase receptor type 2 polypeptide 1	Promoter
chr8	84320000	84320200	Heterogeneous nuclear ribonucleoprotein C-like 2	Transcription Factor Binding
chr8	118205600	118206000	Sterile alpha motif domain containing 12	Promoter Flanking
chr8	140334000	140334200	Trafficking protein particle complex 9	CTCF Binding / Enhancer
chr10	32926000	32926400	Integrin beta 1	Promoter Flanking
chr11	34038200	34038400	Cell Cycle Associated Protein 1	Promoter
chr11	71963400	71963600	Ring Finger Protein 121	Promoter Flanking
chr11	103341600	103341800	Dynein, Cytoplasmic 2, Heavy Chain 1	Enhancer
chr11	121300400	121300600	Sterol-C5-Desaturase	Enhancer
chr13	48327400	48327800	Retinoblastoma Protein 1	Promoter / Enhancer
chr15	48778200	48778400	Centrosomal Protein 152kDa	Enhancer

Chromosome	Peak Start Position	Peak End Position	Gene	Location
chr20	38233400	38233800	KIAA1755 Uncharacterised Protein	Promoter Flanking
chr20	44123000	44123400	Juncophillin 2	Open Chromatin
chr21	31527000	31527200	T-cell Lymphoma Invasion and Metastasis 1	Enhancer

7.12 DNA Binding Motif Discovery

In order for the KCNMA1 protein to bind to the DNA it must harbour a DNA binding motif in the protein sequence. There are various on-line analysis tools which can be utilised to identify these motifs within a protein sequence. GYM is the most recent of the tools, which identifies Helix-Turn-Helix motifs within a protein sequence (Gao, Mathee *et al.* 1999, Narasimhan, Bu *et al.* 2002). The Helix-Turn-Helix motif is one of the most comprehensively studied protein motifs, it has been shown that these HTH motifs are responsible for binding to DNA and the majority of proteins harbouring these motifs are transcription factors.

The KCNMA1 protein sequence was entered into the GYM on-line program and a search for HTH motifs carried out. GYM successfully identified a HTH motif within the KCNMA1 protein sequence, this motif commenced at amino acid 938 of the protein sequence and had a Maximum Blosum score of 31. The sequence was LRQPSI TTTGVNIPIITELVN (Figure 7.8).

Fig. 7.8A MaxiK Protein Sequence with H-T-H Motif

MDALIIPVTMEVPCDSRGQRMWWAFLASSMVTFFGGLFIILLWRTLKYLWTVCCHCGGKT
KKKGRKPRLQGGVIGCMFHLQPREAQKSSSALEVHKKYGDSTGTTLEEAQKINNGSSQAD
GTLKKPVDEKEEAVAAEVGWMTSVKDWAGVMISAQTLTGRVLVVLVFALSIGALVIYFID
SSNPIEESCQNFYKDFTLQIDMAFNVFFLLYFGLRFIAANDKLWFWLEVNSVVDFFTVPPVF
VSVYLNNRSWLGLRFLRALRLIQFSEILQFLNILKTSNSIKLVNLLSIFISTWLTAAGFIHLVN
SGDDPWENFQNNQALTYWECVYLLMVTMSTVGYGDVYAKTTLGRLFMVFFILGGLAMF
ASYVPEIIIELIGNRKKYGGSYSAVSGRKHIVVCGHITLESVSNFLKDFLHKDRDDVNVEIVF
LHNISPPNLELEALFKRHFTQVEFYQGSVLNPHDLARVKIESADACLILANKYCADPDAED
ASNIMRVVISIKNYHPKIRIITQMLQYHNKAHLLNIPSWNWKEGDDAICLAELKLGFIAQSC
LAQGLSTTMLANLFSMRSFIKIEEDTWQKYYLEGVSNEMYTEYLSSAFVGLSFPTVCELCF
VKLKLLMIIAIEYKSANRESRILINPGNHLKIQEGTLGFFIASDAKEVKRAFFYCKACHDDIT
DPKRIKKKCGCKRLEDEQPSTLSPKKKQRNGGMRNSPNTSPKLMRHDPLLIPGNDQIDNM
DSNVKKYDSSTGMFHWCAPKEIEKVILTRSEAAMTVLSGHVVVCIFGDVSSALIGLRNLVM
PLRASNFHYHHELKHIVFVGSIEYLKREWETLHNFPKVSILPGTPLSRADLRAVNINLCDMC
VILSANQNNIIDDTSLQDKECILASLNIKSMQFDDSIGVLQANSQGFTPPGMDRSSPDNSPVH
GMLRQPSITTTGVNIPIITELVNDTNVQFLDQDDDDDPDTELYLTQPFACGTAFAVSVLDSL
MSATYFNDNNILTLIRTLVTGGATPELEALIAEENALRGGYSTPQTLANRDRCRVAQLALL
DGPFADLGDGGGCYGDLFCKALKTYNMLCFGIYRLRDAHLSTPSQCTKRYVITNPPYEFEL
VPTDLIFCLMQQFDHNAGQSRASLSHSSHSSQSSSKKSSSVHSIPSTANRQNRPK

Start Location (Loc): 938
Length of Longest Pattern Matched (LP): 5
Number of Patterns Matched (NPM): 10
Number of Locations Matched: 7
The Maximum blosum score of the whole sequence (Score): 31

Best Motif Sequence:
0123456789 012345678901
LRQPSI TTT GVNIPIITEL VN
^-^---^--- ^^----^--^ --

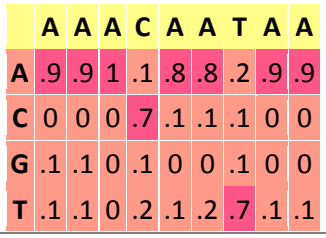
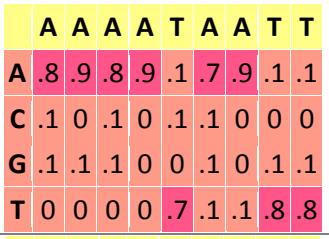
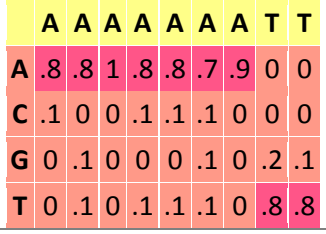
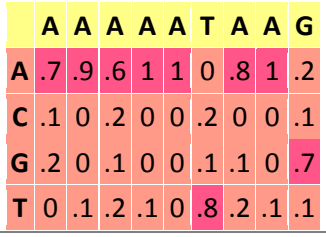
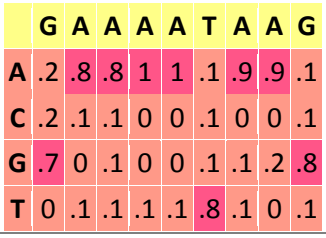
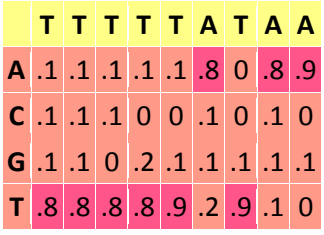
Figure 7.8 Location of Helix-turn-Helix Motif Within the KCNMA1 Protein Sequence
The KCNMA1 protein sequence was entered into the GYM on-line program and a search for HTH motifs carried out. GYM successfully identified a HTH motif within the KCNMA1 protein sequence, this motif commenced at amino acid 938 of the protein sequence and had a Maximum Blosum score of 31. The sequence was LRQPSI TTTGVNIPIITELVN (red).

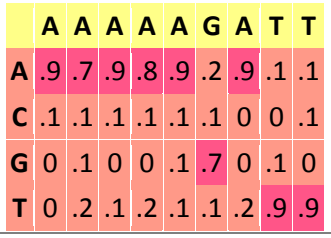
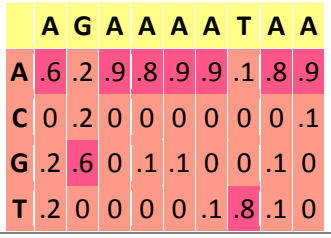
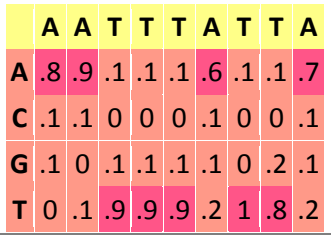
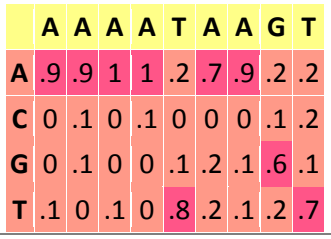
This Blosum score indicates that this sequence has a 31% homology with the accepted HTH motif. This would appear to be quite a low degree of similarity however, LysR a well defined transcriptional regulator, containing a well characterised HTH motif (Maddocks and Oyston 2008) has a Blosum score of 55. It is therefore plausible that this HTH motif in the KCNMA1 C-terminal domain could be responsible for DNA binding. Zinc Fingers and Leucine zippers are alternative structural motifs that facilitate the interaction between protein and DNA however, the KCNMA1 protein was not found to contain either of these motifs (Data not shown).

7.13 KCNMA1 DNA Binding Motif Discovery

As well as a motif in the protein enabling it to bind to the DNA there should also be a motif in the DNA to which the protein binds. To discover any motifs within the peak regions initially the Galaxy Motif Tools > Sequence Logo algorithm was tried, this failed as the sequences were not exactly the same length and there is no functionality within Galaxy to adapt the sequence lengths.

Table 7.9 Motif Discovery in Aligned Enriched Peaks

MOTIF	DETAILS	Probability Matching Matrix
AAACAATAA	Length: 9 Quorum percent: 74% Occurs in 20 input sequences Average #mismatches: 1.6	
AAAATAATT	Length: 9 Quorum percent: 81% Occurs in 22 input sequences Average #mismatches: 1.68	
AAAAAAATT	Length: 9 Quorum percent: 77% Occurs in 21 input sequences Average #mismatches: 1.67	
AAAAATAAG	Length: 9 Quorum percent: 74% Occurs in 20 input sequences Average #mismatches: 1.65	
GAAAATAAG	Length: 9 Quorum percent: 74% Occurs in 20 input sequences Average #mismatches: 1.7	
TTTTTATAA	Length: 9 Quorum percent: 74% Occurs in 20 input sequences Average #mismatches: 1.7	

MOTIF	DETAILS	Probability Matching Matrix
AAAAAGATT	Length: 9 Quorum percent: 74% Occurs in 20 input sequences Average #mismatches: 1.7	
		A A A A A G A T T **A** .9 .7 .9 .8 .9 .2 .9 .1 .1 **C** .1 .1 .1 .1 .1 .1 0 0 .1 **G** 0 .1 0 0 .1 .7 0 .1 0 **T** 0 .2 .1 .2 .1 .1 .2 .9 .9
AGAAAATAA	Length: 9 Quorum percent: 77% Occurs in 21 input sequences Average #mismatches: 1.76	
		A G A A A A T A A **A** .6 .2 .9 .8 .9 .9 .1 .8 .9 **C** 0 .2 0 0 0 0 0 0 .1 **G** .2 .6 0 .1 .1 0 0 .1 0 **T** .2 0 0 0 0 .1 .8 .1 0
AATTTATTA	Length: 9 Quorum percent: 74% Occurs in 20 input sequences Average #mismatches: 1.75	
		A A T T T A T T A **A** .8 .9 .1 .1 .1 .6 .1 .1 .7 **C** .1 .1 0 0 0 .1 0 0 .1 **G** .1 0 .1 .1 .1 .1 0 .2 .1 **T** 0 .1 .9 .9 .9 .2 1 .8 .2
AAAATAAGT	Length: 9 Quorum percent: 74% Occurs in 20 input sequences Average #mismatches: 1.75	
		A A A A T A A G T **A** .9 .9 1 1 .2 .7 .9 .2 .2 **C** 0 .1 0 .1 0 0 0 .1 .2 **G** 0 .1 0 0 .1 .2 .1 .6 .1 **T** .1 0 .1 0 .8 .2 .1 .2 .7

Next the peaks were downloaded and the regions not aligned to known genes were removed then the online Panoptic Motif Search Tool was utilised. Briefly, the sequences were entered into the tool and the parameters adjusted to identify a motif of any length represented in quorum of 75% of the sequences and the program was ran. The program discovered 10 motifs these are detailed in Table 7.9. For the first of these motifs the location of the Motif in the sequence is detailed in Table 7.10.

Table 7.10 Location of Motif 1 in Peak Regions

Sequence Information	Motif Highlighted in Sequence
Seq 1 has 6 matches against Motif 1 AAACAATAA Comment: ***hg19_chr7_121872800_121873000_+ 24.0721701501***	ATTGATTCCACNA1GTACCCATGAGCATGGAATGTTCCA CNA1GATTTGTTTGTGTCCTCTTTTATCACNA1GTTGAGC AGTGGTTTGTAGTTCTCCTTGAAGAGGTCCTTCACATCC CTTGTAAGTTGGACACNA1GTAGGTATTTTATTCTCTTTG AAGCAATTGTGAATGGGAGTTCACCCATGATTTGGCTCT CTGTTTGTCTGTTGT
Seq 2 has 7 matches against Motif 1 AAACAATAA Comment: ***hg19_chr6_90557600_90557800_+ 24.0721701501***	TTGAACTCCTGACCTCAGGTGATCTACCCACCTTGGCCT CCCAAAGTGCTGGAACTACAGGTGTGAGCCACCGTGCC CAGCCCAAAATAGAAATATTTTAAAtAATAtGCAGAAGG TAAACCACNA1GAAACACTGACATAACATTATTATCTCT TTCTTTTAATGATAATCATGCTGTGACCTCATTGAATTTG TTGAGATA
Seq 3 has 8 matches against Motif 1 AAACAATAA Comment: ***hg19_chr5_35748200_35748400_+ 24.0721701501***	CGAAATCAAGATGGAAATTAAAAAATTATTCGAACTGA ATGACAGTGACACAACCTACCAAAACCTCGGATACAGC AAAGGCAGTGCTAAGAGGAAAGTTCATAGCCCTAAATG CCTACATCAAAAAGATCAAAAGAGCACAAACTGACATT CTAAGGTCACACCTCAAGGAACTAGAAAAACAAgAACA AACCAAACCC
Seq 5 has 7 matches against Motif 1 AAACAATAA Comment: ***hg19_chr4_48269400_48269600_+ 24.0721701501***	tgACAATAAGGCAAGCCCAAGCACAGGCGTGTTTGGAG GTGGAGGCAACTACTAGGCCCCCACNA1GTTCTGGGTTG TTTCTTATTTCACNA1GACAAACCTTTTATAATAGCATA ACCACATCGTACAGGATAATTTTACAATTGCCAGTGACC ATGAGCCAATTTCACTTTTCACNA1GAACAGACTTGAAT GCCCAATTTATGATC
Seq 6 has 8 matches against Motif 1 AAACAATAA Comment: ***hg19_chr4_97284600_97284800_+ 24.0721701501***	TTAATTTTTTAAATGGCCAAAAAACTTAAATAGATTCAC NA1GTCCAAAGAAGGTATACAAATGGCCAATAAGCATA TGAAAATGTGCACNA1GTTTGTGAAATGCAAATCAAAA CAAcAATGAGCTATTGTTCATACCTAGTAGGATGGATAT ACTAGAAAAGACAGAGAATAACAAATGTTGATGAGGTT GTGGATAAATTGAA
Seq 7 has 7 matches against Motif 1 AAACAATAA Comment: ***hg19_chr4_105034600_105035000_+ 23.3790313508***	AGATGGTGCCCACCCAGATTGAAGGTGGGTCTGCCTCTC CCAAACCACTGACTCAAATGTTAATCTTCTTTGGCAACA CCTTCACAGACACACCCAGAAACATATTTTGCATTGTTC AATGCAATCAAATTGACACATGAATTAGTCCATTCTCAT GCTGCTAATAAAGACAGGCCCAAGACTCGGTAATTTAT AAGGAAAAGAGGTTTAATGGGCTCACTGCACNA1GATA TGGCTGGGAGACCTCACAATCATGGCAGAAGGCAAAgA AgAAGCAAAGGCACATTTTACATGGCAGCAGGCAAGAG AGCTTTTGCAGGGGAACTCCCATTTATAAAACCATCATA TCTTGTGAGAGTTATTCAGTATCATGAGAACAGTATGGG AGAAAATGCCCCCATGA
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Sequence Information	Motif Highlighted in Sequence
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Seq 10 has 5 matches against Motif 1 AAACAATAA Comment: ***hg19_chr1_1772600_1772800_+ 24.0721701501***	TTACAGGCGTGAGCCACTGCACATGGCCACATTTATTAA TTTTTTGAGACAGATTCTCACTCTGTCACCCTGGCTGGA GTGCAGTGGCATGATCTTGGCTCACTGCAACCTCTGCAT CCCAGGCACNA1GAGCGATTCACNA1GTGCCTCAGCCTC CTGAGTAGCTGGGATTACAGGCACACGCCATGCCTTTTA TTATTTATTAG
Seq 12 has 8 matches against Motif 1 AAACAATAA Comment: ***hg19_chr1_146897000_146897200_+ 24.0721701501***	AGACACAGACTGGCAAATTGGATAAAGAGTCAAGACCT ATCAGTGTGCTGTATTCAGGAAACCCATCTCACGTGCAG AGACACACATAGGCTCAAAATAAAAGGATGGAGGAAG ATCTACCAAGCAAATCGA**AAACAA**a**AA**AAGGCAGGGGT TGCAATCCTAGTCTCTGATAAAACAGACTTTAAACCAAC AAAGATCAA
Seq 13 has 7 matches against Motif 1 AAACAATAA Comment: ***hg19_chr1_216311400_216311600_+ 24.0721701501***	AACAATTTCTTATGAGATATGGAGAATGATATGATTGTA TTGGAATTAGATTGTTATATAT**AAAC**c**ATA**tGTAAAAATT AATCTCATGTTGCTACAGATTATAAGAGTTTTGAAAGCA GATAGACACATCACTAATCTTTGTACACTGGTATTTAGG ATAATGGCCGGTACACAGCAGGCCTCTTAACAAATTGC TGTTC
Seq 14 has 8 matches against Motif 1 AAACAATAA Comment: ***hg19_chr8_84320000_84320200_+ 24.0721701501***	AGTATGCATCACTGCACTCCAGCCTGGAAGACAGAGTG AGACCCTGTCTCAACAAAATAAATTAAAAT**AAA**a**AATA** **A**TATATTTTTCTAACTATCATCCCTTCACNA1GAAATCA GGAACACNA1GCCTTAAGTTCACNA1GTCAATCACNA1G ATGGCAATATCTTTGCATAGATTCATTAAGAATTTGTCC TTTTTAAATAAAAAATATA
Seq 15 has 8 matches against Motif 1 AAACAATAA Comment: ***hg19_chr8_118205600_118206000_+ 23.3790313508***	GAGAGATTTTTATCACCACGAGGCCTGCCCTAAAAGAG CTCCTGAAGGAAGCACTAAACATGGAAAGGAAAAACCG GTACCAGCCACTGCAAAATCATGCCAAATTGTAAAGAC CACCGAGGCTAGGAAGAAACTGCATCAACTAATGAGCA AAATAACCAGCTAACATCATAATGACAGGATCAAATTC ACACATAACAATACTAACCTTAAATGTAAATGGGCTAA ATGCTCAAATTAAAAGGCACAGACTGGCAAATTGGATA AAGAGTCAAGACCCATCAGTGTGCTCTATTCAGGAAAC CCATCTTATGTGCAGAGACACACATAGGCTCAAAATAA AGGGATGGAGGAAGATCTACCAAGCAAATGGA**AAACA** **A**a**AA**AAGGCAGGGGTTGCAAT
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Sequence Information	Motif Highlighted in Sequence
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Seq 20 has 7 matches against Motif 1 AAACAATAA Comment: ***hg19_chr11_71963400_71963600_+ 24.0721701501***	GCTGTGATTACAGGCGTGAGCCACCGTGCTGGCGGGAT GCTTTCTTTAAGGATCACNA1GCAGTCTGGTGGGCACAT GGAAACTTACCTGGGGAGAGGGGGGATTTCTCTTTAGA ATGTCTTAGTTGTTTTTGTAAGACAATGGTACAGAGAAG TCTTGTTGCCACNA1GTTTAAAAAGGGG**AAA**g**AA**a**AA**AC AAAGTTTGACCCT
Seq 21 has 6 matches against Motif 1 AAACAATAA Comment: ***hg19_chr11_103341600_103341800_+ 24.0721701501***	CATACGTGTGCATGTGTCTTTATAGCAGCATGATTTATA ATCCTTTGGGTATATACCCAGTAATGGGATGGCTGGGTC AAATGGTATTTCTAGTTCTAGATCCCTGAGGAATCGCCA CACTGACCACNA1GACAATGGTTGAACTAGTTTACAGTC CCACCAACAGTGTAAAAGCACACNA1GTATTTCTCCACA TCCTCTCCAGC
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Seq 23 has 6 matches against Motif 1 AAACAATAA Comment: ***hg19_chr10_32926000_32926400_+ 23.3790313508***	GTTGTTTGTTTTTTTCTTGTGAATTTGTTTGAGTTCATTG TAGATTCTGGATATTAGCCCTTTGTCAGATGAGTAGATT GCAAAAGTTTTCTCCCATTCTGTAGGCTGCCTGTTCACT CTGATGGTAGTTTCTTTTGCTGTGCAGAAGCTCTTTAGTT TAATTAGATCCTATTTGTCAATTTTGGCTTTTGTTGCCAT TGCTTTTGGTGTTTTAGACATGAAGTCCTTGCCCATGCC TATGTCCTGAATGGTATTGCCTAGGTTTTCTTCTAGGGTT TTTATGGTTTTAGGTCTAATGTTTAAGTATTTAATCCATC TTGAATTAATTTTTGTATAAGGTGTAAAGAAGGGATCCA GTTTCAGCTTTCTCCATATGGCTAGCCAGTTCACNA1GC AGCACCAT
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Sequence Information	Motif Highlighted in Sequence
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Seq 26 has 7 matches against Motif 1 AAACAATAA Comment: ***hg19_chr2_222789400_222789600_+ 24.0721701501***	TCTGTCTAGTATGTTTCTCATTGCAGCTGGCTCCTTCTAA ACCACTGCACAAGACACTTTTGGTGGTTATTATAGCTGT TTGTTTCTTgAAaAATAATGGAAACCATGTGATCCAAAA ACCTCTTTGCTAGTAAACAAAGGCATTCATTGTATCTAT GAAGCAGGCTGTTTTTTAACTTAAAAAAAAGTTTTGGCT GTAG
Seq 27 has 6 matches against Motif 1 AAACAATAA Comment: ***hg19_chr2_239959600_239959800_+ 24.0721701501***	AGTTCTTTTAATTTTTTTAGTATTTATTGATCATTCTTGG GTGTTTCTTG GAGAGGGGGATTTGGCAGGGTCATAGGACAATAGTGGA GGGAAGGTCAGCAGATAAACATGTGAACAAGGGTCTCT GGATCACNA1GTAGGCAGAGGTCCCTGCCGCCCACNA1 GGCAGTGTTTGTGTCCCTGGGTACTTGAGATTAGGGAGT GGC

7.14 Validation of ChIP-seq

To validate the ChIP-seq findings three genes which were identified as enriched with KCNMA1 binding where selected these were; Mitogen Activated Protein Kinase Kinase Kinase 7 (MAP3K7), Retinoblastoma Protein (RB1) and Juncophillin 2 (JHP2). Primers for these genes were designed within the relevant sequences identified during peak calling. PCR was then performed for each of these, utilising the same chromatin as was used in the ChIP-seq analysis but which had not undergone whole genome amplification. Specific enrichment for each of these promoters was seen in the KCNMA1 IP in all three ChIP repeats (Figure 7.9 A, B). This strengthens the case for role of the KCNMA1 C-terminal domain as a protein that also has the ability to bind to DNA.

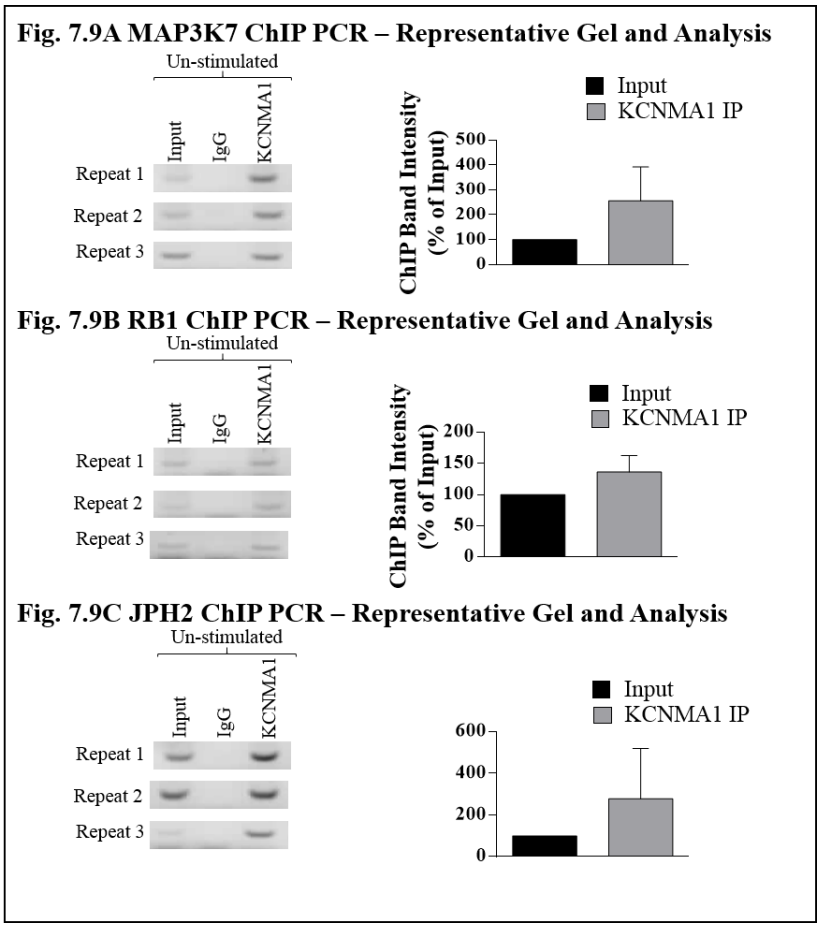

Figure 7.9 Validation of ChIP-seq Results
ChIP chromatin was immunoprecipitated using KCNMA1 c-terminal antisera or non-specific rabbit IgG, the resulting chromatin was then amplified using promoter specific primers. (A) MAP3K7 ChIP PCR. A band was observed in the KCNMA1 IP lane, and no band was observed in the IgG lane indicating that KCNMA1 binds to the MAP3K7 promoter. (B) Rb1 ChIP PCR. A band was observed in the KCNMA1 IP lane, and no band was observed in the IgG lane indicating that KCNMA1 binds to the Rb1 promoter. (C) JPH2 ChIP PCR. A band was observed in the KCNMA1 IP lane, and no band was observed in the IgG lane indicating that KCNMA1 binds to the JPH2 promoter.

7.15 Discussion

7.15.1 The ChIP Assay

There are a number of places where the ChIP assay could be optimised to improve the outcome of the ChIP-seq. Firstly, antibody selection, further characterisation of the primary antibody is needed to confirm its validity for use in the ChIP assay, alternative antibodies could also be trialled and the most suitable selected. Once an appropriate antibody is selected it would then be necessary to optimise the antibody concentration used in the ChIP assay, this first repeat of ChIP-seq has provided information which can be utilised to develop a robust positive control for the KCNMA1 ChIP assay. Development of a robust positive control is necessary prior to further ChIP-seq to build

confidence in the ChIP-seq data and to ensure good quality ChIP chromatin is being used for ChIP-seq and hence improve the quality and reproducibility of the ChIP-seq results.

Secondly, the fragmentation step in the ChIP assay needs improved, although ultrasonic cavitation is effective and adequate for routine ChIP assay protocols it does not produce consistent enough chromatin fragments for ChIP-seq. An alternative approach to ultrasonic cavitation are enzymatic fragmentation using micrococcal nuclease, once optimised this mode of chromatin fragmentation should produce more consistent results. A second alternative is the use of a Bioruptor system. While this also employs ultrasonic cavitation, it is used in a waterbath. This, therefore, ensures more consistent inter-sample temperatures are obtained. Moreover, it allows batch processing of samples, again reducing inter-experimental variation.

The final optimisation of the ChIP concerns the amount of chromatin generated. In this study it was necessary to increase the yield using whole genome amplification. It is possible that this extra step was the reason that the first ~30 bases of each fragment failed the per base sequence content quality control step. A second issue with using whole genome amplification to increase the yield is that it results in the reduction of the complexity of the library, meaning there are fewer unique sequences in the library. This can result in the same fragment being sequenced repeatedly. Optimisation of the two steps above, antibody and fragmentation, should result in the maximum yield possible, if this is still too low then increasing the input DNA may be a preferable step rather than amplifying the resulting chromatin.

7.15.2 ChIP-sequencing

Optimisation of the ChIP protocol will have the effect of improving the ChIP-seq results even before optimisation of the ChIP-seq protocols themselves. There are still a number of areas which could be optimised in the ChIP-seq. Firstly the library preparation, there were experimental problems with the initial kit used as it lacked the adapters required for the library preparation and so it was necessary to switch to a second kit. This switch may have resulted in a less efficient library preparation. The successful addition of the adaptors is necessary as these then bind to the flow cell and allow the fragment to be sequenced. If the library preparation is inefficient this will reduce the number of fragments available

for sequencing. Second, is the optimisation of the amount of DNA loaded into the flow cell, two concentrations were tried in this study 10pM in lane 5 and 8pM in lane 6. Lane 5 was more effective and so further optimisation of this could be carried out.

During library preparation different adapters were added to each end of the fragments then during the sequencing run paired end reads were carried out. This effectively means the fragments were firstly sequenced using a primer for one of the adaptors, then secondly using a primer for the second adaptor. This means the fragment gets sequenced from both ends hence paired-end reads. The principle behind this is that using the two sequence fragments generated and the size of the space between them (calculated from the length of the total fragment) will enable more accurate alignment with the genome. In this study, however, there was a clear difference in the quality of the two paired end reads, the forward reads passed the quality control checks whilst the reverse reads failed a large number of the checks. There are a number of reasons why this could happen, firstly over-clustering of the adapter ligated DNA on the flow cell surface, this is unlikely in this study as the number of clusters was quite low. Secondly, degraded read-2 sequencing primer this is a possibility, however, the other flow cell lanes did not suffer with the same degree of difference between the two paired end reads. Thirdly, low diversity in 3' end of library fragments introduced by library preparation method. This is the most likely source of the problem as there were issues with the library preparation. Again this will need to be optimised prior to any further experimentation.

7.16 Conclusion

As discussed above there are a number of areas which need optimisation in order to build confidence in the data, however, there is strong evidence to support the theory that the KCNMA1 protein appears to have a role in regulating transcription of specific myometrial genes.

Chapter 8: Discussion

8.1 Discussion

Throughout this thesis, a model system, consisting of isolated human myometrial cells grown in culture, has been utilised. The benefit of this system is that smooth muscle-specific changes can be elucidated without other cell types reducing the measureable impact of these changes. However, as a consequence of isolating these myometrial cells the complexity of the signalling pathways seen *in vivo* will have been reduced. Specifically, signalling from both the fetus and surrounding myometrial tissues will have been lost. It is difficult to quantify what affect this loss of input will have on the changes seen within the myometrial cell cultures. In addition to this it is also pertinent to note that only one cytokine, TNF, was utilised within this study. As has been explained previously at the onset of parturition there is an increase in SP-A, MMPs, prostaglandins, TNF, IL1β and IL6, all of which may affect the contractility of the myometrium and hence may have an effect on the KCNMA1 potassium channels, CACNA1Cs and CACNA1Gs. However, this limitation can be addressed in future studies where different combinations of these factors can be utilised and their effect on the KCNMA1 potassium channels, CACNA1Cs and CACNA1Gs examined. These limitations need to be taken into consideration when extrapolating from this data to explain the regulation of parturition.

8.2 The Biological Significance of the Observed Changes in the KCNMA1, CACNA1C and CACNA1G Channels.

A number of the changes un-covered in this study, although statistically significant, were numerically small and this may lead one to question their biological relevance. This is a complex question and must be addressed in the context not only of the protein involved but also the downstream pathways which are subsequently activated and the effects on other associated proteins.

Firstly, it is necessary to understand the difference between statistical significance and biological relevance. Every scientific experiment starts with a null hypothesis, which states: *There is no difference between the groups*. The purpose of any statistical test is to measure the probability (p) of observing an effect given that the null hypothesis is true. The probability is termed the p-value. If the p-value is small it means the results are statistically significant and the null hypothesis can be rejected i.e. there is a difference

between the groups. Biological relevance on the other hand is a measure of the importance of the difference. It can be defined as a meaningful change in the state of the cell, tissue or organ under study.

In the context of this thesis, which seeks to understand the regulation of myometrial contractility, an increase in contraction would be considered a meaningful change in the state of the cells under study and therefore biologically relevant. Therefore, as the small statistical changes in the KCNMA1 channel, CACNA1C and CACNA1G following exposure to TNF, correlated with an increase in the contractility of the cells they meet the criteria to be considered biologically relevant.

8.3 The Role of the KCNMA1, CACNA1C and CACNA1G Channels in Myometrial Cells.

The majority of previous research has focused on a single aspect (either DNA or RNA or protein or electrophysiology) of a single channel, whereas, this thesis has sought to examine multiple aspects (DNA, RNA, protein, localisation and interactions) of multiple channels (KCNMA1, CACNA1C and CACNA1G). In doing so, this research, has revealed novel potential links between the different aspects of the KCNMA1 channel, CACNA1C and CACNA1G, and these are discussed below.

8.3.1 The Relationship Between KCNMA1 Channel Splice Variant Expression and Protein Expression

Previous research by Knaus *et al.* determined that the various protein fragments detected when western blotting for the KCNMA1 channel were by-products of proteolytic cleavage which occurred as a result of the purification process (Knaus, Eberhart *et al.* 1995). However, examination of the western blotting and splice variant data together suggests that the presence of these KCNMA1 fragments could be related to the expression of specific splice variants.

Examination of the KCNMA1 protein sequence suggests that the 50kDa protein fragment detected will still contain a pore and, as the C-terminal region is missing, it could have decreased calcium and voltage sensitivity (Figure 8.1 A and B). This fragment was localised to the membrane, as shown in the western blots, if it is generated *in vivo* rather

than as a result of the extraction process it would alter the gating kinetics of the KCNMA1 channels and hence the contractility of the cell. At splice site M2 the data revealed that the cultured myometrial cells express exons 11 and 12. The use of exon 11 introduces a premature stop codon (Miranda-Rottmann, Kozlov *et al.* 2010), this stop codon results in the truncation of the protein. Given the sequence data it is possible to predict that this truncated protein would be approximately 50kDa in size (Figure 8.1 A). Therefore the presence of this variant could be correlated with the expression of the 50kDa KCNMA1 protein fragment.

Examination of the putative protein sequence for the 65kDa KCNMA1 protein fragment detected suggests that it would also contain the pore region, but would contain a larger portion of the C-terminal region than the 50kDa fragment above. This protein fragment will contain the regulator of potassium conductance domain 1 (RCK1; Figure 8.1 A and C). The full length KCNMA1 channel contains two RCK domains, these RCK domains are thought to have a role in the calcium dependant activation of the KCNMA1 channel. The RCK2 has been shown to have a higher affinity for calcium than RCK1 (Yusifov, Javaherian *et al.* 2010). Therefore as this 65kDa fragment does not contain the RCK2 domain it could have decreased calcium sensitivity. At splice site 3, the entire of exon 19 was shown to be utilised, this variant was only expressed at a low level similar to the expression of the 65kDa protein fragment. The utilisation of exon 19 also results in a decrease in the calcium sensitivity of the channel. The similarities in the putative electrophysiological effects and the expression patterns of the 65kDa protein fragment and the splice site 3 variant would suggest that these two are linked. In addition to this, given the location of splice site 3, it is possible to speculate that if exon 19 contained either a stop codon or a proteolytic cleavage site the protein generated would be approximately 65kDa (Figure 8.1 A). This putative link between splice site 3 and the 65kDa fragment is worthy of more in-depth research.

Finally, examination of the putative protein sequence for the 90kDa KCNMA1 fragment detected, suggests that it will contain the pore and also both the RCK1 and RCK2 domains (Figure 8.1 A and D). This should result in an increase in the calcium responsiveness of the resulting channel compared to the 65kDa fragment. At the same time the M6 variant containing exon 29 was expressed, variants containing exon 29 have been shown to have increased activation rates and open more rapidly upon increases in intracellular calcium.

The expression of this variant appears to correspond to the 90kDa protein found within the nuclear fractions. Therefore, the increased activation rate of the M6 variant containing exon 29 may be as a result of the loss of the inhibitory C-terminal tail region.

One way to more clearly define these putative links between splice variant and protein fragment expression would be to clone the KCNMA1 protein into an expression vector and selectively express the splice variants of interest. The KCNMA1 protein subsequently produced could then be characterised.

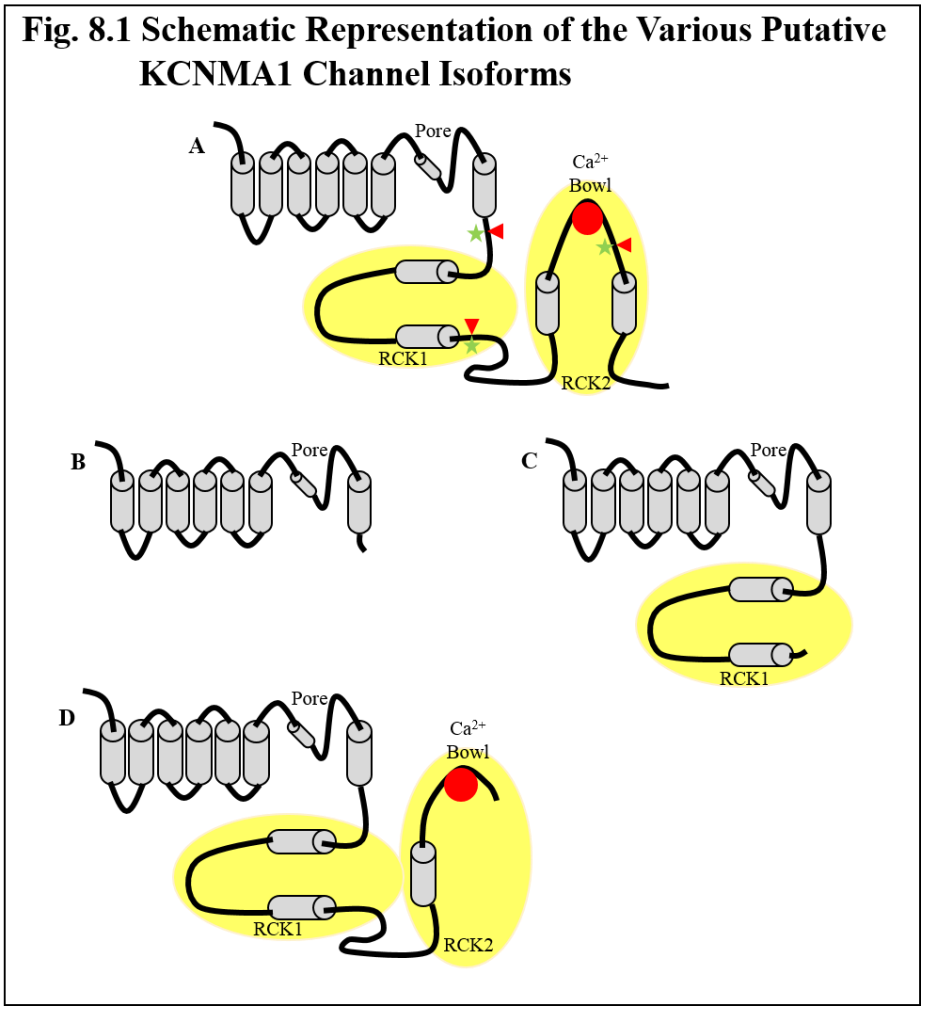

Figure 8.1 Schematic Representation of the Various Putative KCNMA1 Isoforms
Diagram of the KCNMA1 channel a-subunit, showing the seven membrane spanning domains, with a pore region (Pore). The long cytoplasmic tail contains four hydrophobic domains. The regulator of potassium conductance (RCK) 1 and RCK2 are highlighted by yellow shading. The Ca^{2+} bowl (Red circle) is a high-affinity Ca^{2+} binding site that lies within RCK2. **(A)** Full length KCNMA1 channel. Splice sites putatively associated with the shorter isoforms are highlighted (red triangles) and the putative end of the various KCNMA1 protein fragments are highlighted (green stars). **(B)** 50kDa KCNMA1 fragment, **(C)** 65kDa KCNMA1 fragment and (D) 90kDa KCNMA1 fragment.

8.3.2 The Relationship Between CACNA1C Splice Site 4 Variant Expression and Acetylation

This research has demonstrated that CACNA1C exon 8 is only expressed following exposure to TSA. Suggesting that acetylation has a role in the expression of this particular splice variant.

Previous research has demonstrated that nucleosomes, DNA methylation and histone modifications are enriched on exons (Zhou, Luo *et al.* 2014) and this enrichment has been linked to splicing regulation (Hnilicová and Staněk 2011). It was noted that hyperacetylation localised to the region surrounding an alternative exon resulted in the skipping of this exon (Zhou, Hinman et al. 2011). Furthermore, HDAC inhibition has been shown to affect the splicing of ~700 genes (Hnilicová, Hozeifi *et al.* 2011), one way in which this is thought to occur is through an increase in histone H4 acetylation and subsequently an increase in RNA polymerase II processivity. It is thought, due to the rapid nature of histone acetylation and deacetylation, that acetylation preferentially modulates the alternative splicing of genes that respond rapidly to changing cellular conditions (Hnilicová and Staněk 2011). Recently, TSA has been shown to affect alternative splicing, this is thought to be through it induction of a more relaxed chromatin state which results in the redistribution of multiple splicing factors (Kadener, Cramer et al. 2001, Karolczak-Bayatti, Sweeney et al. 2011, Schor, Llères et al. 2012).

Utilising the ChIP assay it would be possible to determine if increased acetylation of the region surrounding CACNA1C exons 8 and 8* was involved in the inclusion or exclusion of a particular exon.

8.3.3 The Relationship Between CACNA1G Splice Variant Expression, Protein Expression and Localisation.

As with both the KCNMA1 and CACNA1C, different sized CACNA1G proteins were detected on the western blots. Two forms of the CACNA1G have been defined by Monteil *et al.*, a short version termed CACNA1G $\alpha_{1G\text{-}b}$ and a longer version termed CACNA1G $\alpha_{1G\text{-}a.}$ These two forms were shown to contain distinct III-IV loops, $\alpha_{1G\text{-}b}$ is formed utilising an alternative 5' splice donor site in exon 25 combined with the acceptor site on exon 27 (Monteil, Chemin *et al.* 2000). The splice variant analysis revealed that the

primary myocytes express exons 25 and 26 this would account for the longer form of the CACNA1G. However, there was no alternative variant detected in the RNA, which lacked exon 26 that could account for the shorter CACNA1G channel protein detected. Therefore, it would appear that the shorter 249kDa channel detected in this study must have arisen via a different mechanism. Combining the protein data and RNA splicing data revealed that the short CACNA1G isoform terminates in a splicing region. The splice site in question involves a splice donor site on exon 30 (30B) and a splice acceptor site on exon 31 (31A; Figure 8.2 A and B).

Fig. 8.2 Schematic Representation of the Putative TTCC Channel Isoforms

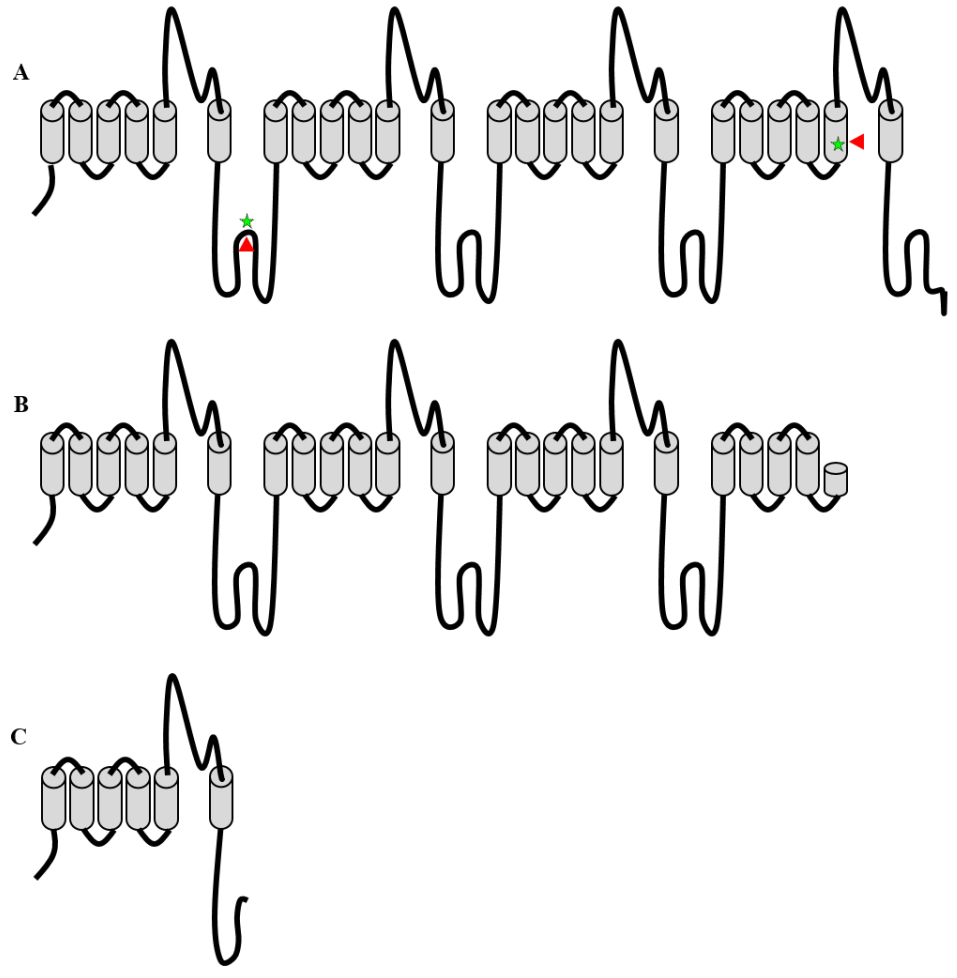

Figure 8.2 Schematic Representation of the Putative CACNA1G Isoforms
Diagram of the CACNA1G channel a-subunit, showing the membrane spanning domains, and linking loops (A) Full length CACNA1G channel. Splice sites putatively associated with the shorter isoforms are highlighted (red triangles) and the putative end of the various CACNA1G protein fragments are highlighted (green stars). (B) 249kDa CACNA1G fragment, (C) 69kDa CACNA1G fragment.

Exon 30B has been shown to affect the voltage dependant activation of the channel while exon 31A was shown to result in the premature truncation of the channel (Emerick, Stein *et al.* 2006). This, however, does not seem to fit with the data presented here. Within this study exon 31A was constitutively expressed whereas exon 30B was expressed at different levels dependant on the stimulation. However both long and truncated CACNA1G were present in un-stimulated samples and following both TNF and TSA stimulation. This may suggest that exon 31A is not linked to the 249kDa isoform or it may suggest that the truncation of the channel is due to proteolytic cleavage rather than the introduction of a stop codon. This proteolytic cleavage may therefore not be a constitutive event but rather a controlled event driven by the needs of the cell.

A final point to note is the observation that membrane localisation of the CACNA1G seems to be promoted by both TNF and TSA. Previous research has identified splice site 1 variants as having a role in trafficking of the protein (Shcheglovitov, Vitko *et al.* 2008). This site involves the insertion or deletion of exon 8 and unfortunately was not examined in this study. However, there was a 69kDa CACNA1G protein fragment detected, and this fragment terminates within splice site 1 (Figure 8.2 A). It is possible, therefore, that either insertion or deletion of exon 8 results in the truncation of the CACNA1G (Figure 8.2 C) and hence affects the membrane trafficking of the CACNA1G protein. Future work should involve examining the expression of this variant within these primary myocytes in order to determine if changes in splice variant expression at this location are responsible for the changes in either membrane localisation or premature truncation of this channel.

8.4 The Role of the KCNMA1, CACNA1C and CACNA1G Channels in the Generation of Relaxation in Cultured Myometrial Cells

During pregnancy the myometrium is quiescent allowing it to expand to accommodate the growing fetus. TSA, a broad spectrum deacetylase inhibitor, has been shown to cause relaxation in myometrial smooth muscle strips (Carvajal, Germain *et al.* 2000, Moynihan, Hehir *et al.* 2008, Webster, Waite *et al.* 2013) and in this study it was seen to cause relaxation of the primary myometrial cells grown in culture. Therefore, it was decided to use TSA to simulate the relaxed state of the myometrium during pregnancy.

8.4.1 The KCNMA1 Channel

TSA stimulation resulted in a significant decrease in KCNMA1 channel mRNA, however, this coincided with an increase in the amount of KCNMA1 protein. This discrepancy between the mRNA levels and protein levels could be as a result of an increase in translation from a cytoplasmic store of KCNMA1 channel mRNA which would result in the observed decrease in RNA and concurrent increase in protein levels. Alternatively, the increase in protein could be due to either an increase in retention of the KCNMA1 channel or a decrease in the degradation of the KCNMA1 channel. Research has demonstrated that the KCNMA1 channel undergoes ubiquitination (Kyle and Braun 2014) and separate research has demonstrated that acetylation can protect proteins from ubiquitination (Caron, Boyault *et al.* 2005). Therefore the up-regulation in KCNMA1 protein could be as a result of TSA inhibition of lysine de-acetylation and subsequently the decreased ubiquitination of the KCNMA1 channel. This theory could be tested by measuring the turnover of the KCNMA1 protein. Interestingly, the observed increase in KCNMA1 channel protein was confined to the cytoplasmic fraction indicating that two separate mechanism exist, one to trigger the translation of the protein and a second to trigger its translocation to the membrane. This delay in trafficking of the KCNMA1 protein to the plasma membrane has been demonstrated by Kim *et al.* in chick cochlear development (Kim, Beyer *et al.* 2010). Further studied to identify the trigger responsible for trafficking the KCNMA1 channel to the plasma membrane would be an important next step in this research.

Following TSA stimulation there was an association between the membrane localised KCNMA1 channel and Cav-1 indicating that a portion of the KCNMA1 channels are putatively housed within caveolae. There was an association between the KCNMA1 channel and both $G_{\alpha s}$ and the β_2-AR. The inconsistency of the detected association with $G_{\alpha s}$ may be as a result of the transient nature of its interactions. In future work it would be pertinent to stabilise these transient interactions prior to co-localisation studies as this will give a more accurate measure of its interaction with the KCNMA1 and CACNA1C. The association with the β_2-AR may indicate that the KCNMA1 channel is being regulated by the β_2-AR under these conditions.

8.4.2 The CACNA1C Channel

Following TSA stimulation, CACNA1C channel mRNA was unchanged and this corresponded to a small decrease in the expression of both the full length and C-terminally truncated CACNA1C protein isoforms. The membrane-localised CACNA1C channel was found associated with both $G_{\alpha s}$ and the β_2AR indicating indicating that they may be regulating the CACNA1C channel under these conditions. The observed association between $G_{\alpha s}$ and the CACNA1C would indicate that activation of the CACNA1C is occurring via the cAMP independent pathway through the direct interaction of $G_{\alpha s}$ with the CACNA1C (Yatani, Imoto *et al.* 1988). The CACNA1C was also found associated with Cav-1 under these conditions. Localisation of the CACNA1C with caveolae has been linked to both calcium signalling to the nucleus via the NFAT transcription factor (Makarewich, Correll *et al.* 2012), the production of calcium sparks and possibly calcium waves (Daniel, El-Yazbi *et al.* 2006).

CACNA1C splice variant analysis also revealed the novel expression of exon 8, the inclusion of this exon results in channels with higher DHP sensitivity, slower activation and more rapid deactivation kinetics meaning that they are less supportive of contraction (Soldatov 1992). There was also an overall reduction in the level of splice variants expressed in the C-terminal region of the channel. This observation may indicate that the majority of the channels are truncated which would increase their ion conductance, or this may be an artifactual result due to the significant reduction in the overall channel.

8.4.3 The CACNA1G Channel

In the TSA-stimulated myocytes there was no significant change in CACNA1G channel mRNA and no significant change in the expression of the CACNA1G protein. CACNA1G was, however, detected within the membrane fraction suggesting it was inserted therein. This observation suggests that TSA supports the membrane localisation of the CACNA1G rather than inducing an increase in expression of the CACNA1G protein. Membrane-localisation of the CACNA1G has been shown to be enhanced by activation of the Ras-ERK pathway (Mor, Beharier *et al.* 2012), therefore, the observed increase in membrane localised CACNA1G may be as a result of the action of TSA on this pathway. A second possibility is that acetylation of the CACNA1G may promote its membrane localisation. Further study of the activation of the Ras-ERK pathway in the

context of CACNA1G membrane localisation and the myometrium would be a logical next step in this research.

8.4.4 The Combined Role of the KCNMA1, CACNA1C and CACNA1G Channels in the Generation of Relaxation in Cultured Myometrial Myocytes

Within the relaxed myometrial cells, the KCNMA1, CACNA1C and CACNA1G channels are transcribed at a low level and a proportion of both the KCNMA1 and CACNA1C channels were detected associated with Cav-1 and hence with caveolae. The splice variants expressed by the KCNMA1 channel; Mk44 +132bp, M2 (+exons 11 and 12), M3 (STREX-2) and M5 (+exon 29), are less responsive to both calcium and voltage. However, these are expressed alongside CACNA1C isoforms harbouring slower activation and more rapid inactivation kinetics, as well as proteolytically cleaved CACNA1Gs which lack the C-terminal tail region. This region of the CACNA1G has been shown to be responsible for the rate of inactivation of the channel (Staes, Talavera *et al.* 2001), therefore, inclusion of exon 31A which can result in premature truncation of the protein may alter the inactivation of the channel. Overall this would result in cells which are less responsive to calcium and voltage and hence are less contractile.

8.5 The Role of the KCNMA1, CACNA1C and CACNA1G Channels in Un-stimulated Cultured Myometrial Myocytes

8.5.1 The KCNMA1 Channel

The myometrial cell cultures were derived from term pregnant, non-labouring biopsies and therefore un-stimulated samples in this study represent this stage of pregnancy, just prior to the onset of contractions. In the un-stimulated samples, there is evidence for both full length and C-terminally truncated KCNMA1 channel protein within the membrane fraction suggesting they were inserted therein, at the same time there is membrane localisation of the 50kDa N-terminal fragment. The KCNMA1 channel mRNA is also increased compared to that seen in the TSA-stimulated myometrial cell cultures. These membrane-localised channels were shown to associate with Cav-1 and therefore may exist within caveolae. This association would enable the KCNMA1 channel to respond rapidly to changes in membrane potential and calcium signalling. In addition, these KCNMA1 channels also were shown to associate with the β_2AR and $G_{\alpha s}$. Co-localisation

of the KCNMA1 channel and $G_{\alpha s}$ would suggest that $G_{\alpha s}$ is directly activating the KCNMA1 channel. As the direct activation of the KCNMA1 channel by $G_{\alpha s}$ is a highly localised membrane-delimited process (Forscher, Oxford *et al.* 1986, Dolphin 2003). Although it is pertinent to remember that co-localisation can indicate that either two proteins interact directly or interact via one or more bridging molecules. As explained for the CACNA1C previously, this putative direct interaction between $G_{\alpha s}$ and the KCNMA1 channel would indicate that activation of the channel is occurring in a cAMP-independent manner (Kume, Graziano *et al.* 1992). This putative direct interaction between the KCNMA1 channel and $G_{\alpha s}$ may also indicate that activation of the KCNMA1 channel by the β_2AR is also occurring via a cAMP independent pathway (Scornik, Codina *et al.* 1993, Kume, Hall *et al.* 1994, Tanaka, Yamashita *et al.* 2003, Tanaka, Horinouchi *et al.* 2005), as the β_2AR activates the KCNMA1 channel via $G_{\alpha s}$ (Tanaka, Yamashita *et al.* 2003, Tanaka, Horinouchi *et al.* 2005). When $G_{\alpha s}$ activates the KCNMA1 via the cAMP pathway, $G_{\alpha s}$ directly interacts with AC not the KCNMA1 channel.

The STREX-2 variant was found to be utilised in the channel at this juncture, the effect of this variant is dependent on the level of PKA in the tissue and the subsequent phosphorylation of STREX-2 by PKA. Therefore, as the levels of PKA rise towards the onset of parturition, there may be an increase in STREX-2 phosphorylation. This increased STREX-2 phosphorylation will then support the contractile phenotype of the cells (Europe-Finner, Phaneuf *et al.* 1994, Europe-Finner, Phaneuf *et al.* 1997, Zhou, Wang *et al.* 2000, Zhu, Eghbali *et al.* 2005). However, as explained above the co-immunoprecipitation data suggest that at this juncture the activation of the KCNMA1 channel is at least partially cAMP-independent. Although the precise mechanism by which $G_{\alpha s}$ directly activates the KCNMA1 channel has not been elucidated it does not appear to involve phosphorylation of the channel (Brown and Birnbaumer 1988). Therefore, PKA phosphorylation may have a reduced role in activating the KCNMA1 channel which may in turn lead to a reduction in the phosphorylation of the STREX-2 variant. This could mean that the contractile effect of the phosphorylated STREX-2 variant may also be reduced. Finally, the KCNMA1 channel RNA also contained exon 29, this variant once again affects the channels calcium sensitivity. This appears to be due to the introduction of a cleavage site which, results in the cleavage of the C-terminal region decreasing the calcium sensitivity of the channel and possibly creating the 90kDa fragment observed in the western blots.

8.5.2 The CACNA1C Channel

The CACNA1C protein is also present, however in the un-stimulated samples it is predominantly contained within the cytoplasm. The portion of the channel housed within the membrane is again associated with both caveolae via Cav-1 and the β_2AR, although unlike the KCNMA1 channel it is possible that these represent two distinct complexes rather than one multiprotein complex. At this juncture the CACNA1C does not appear to co-localise with $G_{\alpha s}$. This lack of association may be due to the transient nature of $G_{\alpha s}$ interactions or it may represent a switch in the pathway of CACNA1C activation to a cAMP-dependent pathway.

As with the KCNMA1 channel, both full length and C-terminally truncated channel forms were detected within the membrane fraction, suggesting they were inserted therein, and also within the cytoplasm. Splice variant analysis reveals the presence of exon 8* which promotes calcium influx, and hence would support contraction. There were two variants detected at splice site 10, a longer version containing exons 30, 31, 32 and 34 and a shorter form containing exons 30, 31, 33 and 34. The longer form has increased excitation-contraction coupling (Huang, Xu *et al.* 2006) in comparison to the shorter form. The presence of both these variants within the myometrial cell cultures could provide a balance to each other, one supporting contraction and one supporting relaxation. Finally, at splice site 11/12 only one variant was present containing exons 40, 41, 42 and 43 without any insertions are deletions, cleavage of the C-terminal region of the channel occurs in the region of this variant. The lack of alternative variants in this region would indicate that the presence particular splice variants does not drive the cleavage of the channel, instead there must be an alternative mechanism causing the cleavage of the C-terminal tail region.

8.5.3 The CACNA1G Channel

The CACNA1G was not detected within the membrane fraction at this stage, suggesting it is not inserted therein and so will not be contributing to the excitability of the cells, however, two forms were detected within the cytoplasm.

Splice variant analysis of the CACNA1G reveals, exon 14 is not present, exon 25 is present without any insertions, as is exon 26, these variants will have the effect of producing functional channels with midpoint activation rates (Bertolesi, Walia Da Silva *et al.* 2006). Finally, in splice site 7/8 there are two variants expressed one containing exons 30B and 31A and one containing just exon 31A. Exon 31A has been shown to result in prematurely truncated protein (Emerick, Stein *et al.* 2006) and may account for the shorter protein detected within the cytoplasm.

Following ChIP analysis, binding of the CACNA1G promoter by CBP, SP1 and RelA was observed. There was no evidence of enrichment of the promoter with p50 and so a different NFκB subunit, possibly RelA homodimers, appear to be responsible for this regulation. The presence of CBP may again indicate that an enhanceosome like complex is involved in the basal transcription of this channel.

8.5.4 The Combined Role of the KCNMA1, CACNA1C and CACNA1G Channels in Un-stimulated Cultured Myometrial Cells.

Taken together, the data above reveals a picture of cells primed to contract. There are KCNMA1 channels with decreased calcium sensitivity housed in complexes to minimise the effect of this decreased sensitivity. There are CACNA1C with increased ion conductance capabilities which are housed in complexes to maximise the efficiency of calcium ion signal transduction. Finally, there are CACNA1G prepared in the cytoplasm ready to be inserted into the membrane to regulate the timing and force of the contractions (Figure 8.3).

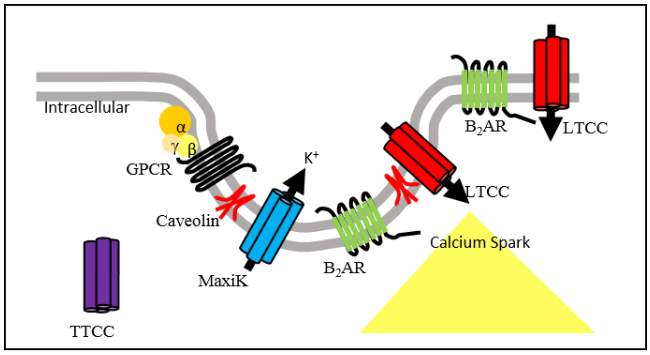

Figure 8.3 Schematic Representation of the KCNMA1 channel, CACNA1C and CACNA1G location and associations prior to parturition
CACNA1C – CACNA1C calcium channel; GPCR – G protein coupled receptor, α – G protein alpha subunit, β – G protein beta subunit, γ - G protein gamma subunit, CACNA1G – CACNA1G calcium channel

8.6 The Role of the KCNMA1, CACNA1C and CACNA1G Channels in the Generation of Contraction in Cultured Myometrial Cells

The onset of parturition is characterised by an influx of cytokines including TNF, IL-1β and IL6 (Romero, Mazor *et al.* 1992, Opsjln, Wathen *et al.* 1993, Keelan, Marvin *et al.* 1999, Osman, Young *et al.* 2003). Within this study, TNF stimulation was shown to cause significant contraction of the myometrial cells grown in culture. Therefore, it was decided to use TNF to simulate the contractile state of the myometrium during parturition.

8.6.1 The KCNMA1 Channel

Following TNF stimulation, there was a significant increase in the transcription of the KCNMA1 channel. However, there was no corresponding increase in membrane-localised full length KCNMA1 channel. Kim *et al.* demonstrated this lag between KCNMA1 RNA and protein up-regulation in chick cochlear development and determined that it was due to delays in protein synthesis and trafficking / scaffolding of the channel subunits (Kim, Beyer *et al.* 2010) a similar delay may be occurring in the myometrium. There was, however, an increase in the membrane localisation of the 50kDa KCNMA1 fragment.

This increase in the 50kDa fragment was not associated with an increase in the expression of the splice site 2 variant containing exons 11 and 12. This would indicate that although the presence of this variant may permit the proteolytic processing of the channel, a different mechanism is in place to regulate this processing. This membrane-localised KCNMA1 channel is found associated with $G_{\alpha s}$ and the β_2AR demonstrating that the KCNMA1 channel is likely to be active. There is an association between the KCNMA1 channel and Cav-1 indicating that the KCNMA1 channel is likely to be housed within caveolae meaning that its surface expression will be reduced.

Splice variant analysis demonstrates that TNF does not cause a significant change in the splice variants of the KCNMA1 channel being expressed with the exception of the exon 19 variant. The exon 19 variant is significantly reduced and this coincides with the reduction in the 65kDa N-terminal fragment supporting the theory that this variant and protein fragment are linked. This may have the result of increasing the calcium sensitivity

of the KCNMA1 channel. Although there is no change in the expression of the STREX-2 variant, the role of this variant will alter as parturition progresses. Early in parturition, as the $G_{\alpha s}$ levels are high, the levels of PKA will be correspondingly high and hence this variant will support contraction (Europe-Finner, Phaneuf et al. 1994, Europe-Finner, Phaneuf et al. 1997). As parturition progresses, levels of $G_{\alpha s}$ and PKA will decrease and hence the support of contraction by the STREX-2 splice variant will also decrease, this switch may have a role in the termination of parturition.

8.6.2 The CACNA1C Channel

Following TNF stimulation there was a significant increase in the transcription of the CACNA1C and this coincided with a significant increase in the membrane localisation of C-terminally truncated channel. This membrane localised channel was found associated with $G_{\alpha s}$ and the β_2AR indicating that, like the KCNMA1 channel, it is also likely to be active. There is also an increase in association with Cav-1 indicating that more CACNA1C is housed within caveolae after TNF stimulation. Localisation of the CACNA1C within caveolae will improve the efficiency of calcium signal transduction and increase excitation-contraction coupling.

Splice variant analysis again shows that TNF does not cause any significant changes in the splice variant profile of the CACNA1C.

8.6.3 The CACNA1G Channel

TNF stimulation had no effect on the transcription of the CACNA1G. There was, however, a significant increase in the membrane localisation of this channel, demonstrating that TNF may promote the insertion of this channel into the membrane where it can then regulate the timing and force of contractions. TNF did, however, significantly increase the expression of the splice site 2 variant without exon 14. The omission of this exon will result in the loss of a PKC phosphorylation site. PKC phosphorylation has been shown to activate the CACNA1G (Park, Kang *et al.* 2006). Therefore, the loss of a PKC phosphorylation site may act to limit the activation of the CACNA1G at this juncture.

TNF also caused an increase in the expression of the splice site 4 variant containing exon 25. This variant results in channels which open more easily at more negative membrane potentials and as such these variants may be key to the ability of the myometrium to generate spontaneous phasic contractions. The generation of these spontaneous phasic contractions is due to the ability of the cell to fire regenerative action potentials (Lee, Ahn *et al.* 2009). Regenerative action potentials are created when a channel opening at a low threshold is able to initiate the opening of enough channels to initiate an action potential. CACNA1Gs with the ability to open at more negative membrane potentials due to the presence of these variants will allow calcium to enter the cell and so will push the membrane potential to more positive potentials which will then allow the CACNA1Cs to open and generate an action potential.

8.6.4 The Combined Role of the KCNMA1, CACNA1C and CACNA1G Channels in the Generation of Contraction in Cultured Myometrial Cells.

It is evident that the splice variant profiles of the KCNMA1 channels and CACNA1C are set prior to the influx of cytokines characteristic of parturition. The effect of TNF is two-fold, firstly it increases the transcription of all the channels and secondly it affects the localisation of the channels. The 50kDa KCNMA1 channel fragment, with decreased calcium sensitivity, is mobilised to the membrane and at the same time membrane-bound KCNMA1 channel is associated with caveolae microdomains. The short form of the CACNA1C channel, with increased ion conductance, is also mobilised to the membrane and there is CACNA1C associated with Cav-1. Finally the full length CACNA1G is also mobilised to the membrane. One would speculate that these changes could result in cells that can counteract changes in calcium signalling less efficiently because the KCNMA1 channels present are less sensitive to calcium. Therefore they respond less efficiently to increasing intracellular calcium. At the same time these cells have a greater ability to contract as they possess CACNA1C with greater ion conductance. These particular CACNA1C isoforms are housed within microdomains which can initiate calcium sparks and more efficient excitation-contraction coupling (Darby, Kwan *et al.* 2000, Daniel, El-Yazbi *et al.* 2006). Finally, these cells are equipped with CACNA1G which can modulate the frequency, force and amplitude of the contractions stimulated by the activation of the CACNA1C.

In summary, this thesis has demonstrated that in myometrial cells generated from term pregnant biopsies the expression and splice variant profile of the KCNMA1, CACNA1C and CACNA1G has already been adapted to support contraction. The influx of cytokines into the myometrial tissue, specifically TNF, then alters the localisation of these channels to optimise the efficiency of calcium signalling and excitation-contraction coupling via the calcium channels and minimise the counteraction of contraction by the KCNMA1 channel. In this way the myometrium is moved from quiescence to contractility.

A further finding from this thesis is the dual role of the KCNMA1 protein as an ion channel and a transcription factor. This transcription factor activity is a further way in which the KCNMA1 protein can regulate the timing of parturition.

8.7 The KCNMA1 Protein as a Transcriptional Regulator

Throughout this thesis it has been demonstrated that the KCNMA1 channel can regulate its response to the changing cellular environment through the use of splice variants, its sub-cellular localisation and its association with other proteins. In this way it can regulate the contractility of the tissue and hence, in terms of this project, it may regulate the timing of parturition. This study has uncovered a further level at which the KCNMA1 protein can regulate the cells responses, through its action as a transcription factor. Although this research needs further optimisation and validation it has provided a fascinating insight into the processes which the KCNMA1 protein can regulate.

8.7.1 Transition Through G1/S Cell Cycle

The ChIP-seq and subsequent ChIP validation PCR experiments revealed that the KCNMA1 protein bound to the promoter/enhancer region of the Retinoblastoma protein (RB1) gene. RB1 is a tumour suppressor protein which has been shown to be dysfunctional in several cancers (Murphree and Benedict 1984). The role of the RB1 protein is to prevent excessive cell growth, this is accomplished by blocking cell cycle progression at the G1/S transition until the cell is ready to divide (Goodrich, Wang *et al.* 1991, Templeton, Park *et al.* 1991, Connell-Crowley, Harper *et al.* 1997). This block in cycle progression is achieved via RB1 binding and inhibiting transcription factor E2F which is needed for S phase gene transcription (Connell-Crowley, Harper *et al.* 1997, Funk and Galloway 1998). Once the cell is ready to divide, RB1 is inactivated by

phosphorylation, the block is released and the cell cycle proceeds. The phosphorylation of RB1 is thought be carried out by G1 cyclin depenant kinases (Cdk) and in particular cyclin D-type (Cdk4) and E-type (Cdk2), phosphorylation of RB1 means RB1 can no longer bind E2F and therefore the inhibition of E2F is removed (Dynlacht, Flores *et al.* 1994, Connell-Crowley, Harper *et al.* 1997).

The uterus, and specifically the myometrium, increases in size by around 20-fold during pregnancy, smooth muscle cell proliferation (hyperplasia) is the key process in achieving this expansion during early pregnancy (Ramsey 1994). RB1 has been shown to be involved in cell cycle progression and proliferation in vascular smooth muscle cells (Li, Duman-Scheel *et al.* 2010) and myometrial smooth muscle cells (Taniguchi, Morita *et al.* 2001).

Later in pregnancy there is a switch and the increase in the capacity of the uterus is then achieved predominantly by an increase in cell size (hypertrophy) this hypertrophy is driven by stretch. At the end of pregnancy and the onset of parturition it has been shown that this stretch induces the expression of both contraction associated proteins and pro-inflammatory cytokines (Ou, Orsino *et al.* 1997, Parry, Bathgate *et al.* 1997, Ou, Chen *et al.* 1998, Wu, Ma *et al.* 1999, Loudon, Sooranna *et al.* 2004) this then tips the balance from the quiescent phenotype seen during pregnancy to the contractile phenotype typical of parturition.

Excess hyperplasia will reduce both hypertrophy and stretch whilst a lack of hyperplasia will result in increased hypertrophy and stretch. A balance, therefore, between hyperplasia and hypertrophy late in pregnancy is necessary for parturition to occur at the right juncture. Regulation of the RB1 protein by the KCNMA1 protein is one way in which this balance could be regulated.

8.7.2 Calcium Homeostasis

The overriding trigger for the onset of spontaneous myometrial contractions is the increase in intracellular calcium. This increase is achieved through a balance between calcium influx, calcium release from intracellular stores and calcium removal (Tribe, Moriarty *et al.* 2000, Tribe 2001). The influx of calcium generates an action potential which then initiates cross-bridge cycling and muscle contraction. The link by which this

change in membrane potential then leads to physical contraction is termed excitation-contraction coupling.

Calcium Sparks are important in this excitation-contraction coupling (Cheng, Lederer *et al.* 1993). Calcium sparks are generated through the activation of a cluster of ryanodine receptors which then cause calcium to be released from the sarcoplasmic reticulum, it is possible that caveolae have a role in the generation of these sparks. Caveolae create microdomains which can house spatially contained increases in calcium, these transient increases can then cause a calcium spark from the closely associated sarcoplasmic reticulum (Cheng, Lederer *et al.* 1993, Löhn, Fürstenau *et al.* 2000).

Juncophillin 2 (JPH2) is a member of a family of junctional membrane complex proteins, these proteins provide a physical link between the plasma membrane and the membranes of the endoplasmic and sarcoplasmic reticulum (Garbino, van Oort *et al.* 2009). This physical association will facilitate the transduction of the calcium signal and hence promote the generation of calcium sparks (Golini, Chouabe *et al.* 2011, Landstrom, Kellen *et al.* 2011).

The KCNMA1 protein was found bound to the regulatory region of the JPH2 gene, regulation of the JPH2 protein will affect excitation-contraction coupling and this in turn will affect the contractility of the tissue. In terms of parturition, regulation of this protein is another level at which the KCNMA1 protein can affect the timing of parturition by fine tuning the cells excitation-contraction coupling.

8.7.3 NFκB Signalling

The process of parturition has been shown involve a controlled inflammatory response. NFκB is a key regulator of the inflammation cascade and therefore NFκB signalling is an important part of the regulation of the parturition process. Mitogen Activated Protein Kinase Kinase Kinase 7 (MAP3K7) is a key protein in the signalling cascade following both TNF and LPS activation of NFκB.

Following TNF stimulation MAP3K7 is rapidly polyubiquitinated leading to its activation (Hamidi, von Bulow *et al.* 2012). Once activated it can have either a stimulatory (Craig,

Larkin *et al.* 2000) or inhibitory (Ajibade, Wang *et al.* 2012) effect on the NFκB pathway. Activated MAP3K7 then acts via IκB Kinase (IKK), which subsequently acts via IκBα which then either retains NFκB in the cytoplasm or releases NFκB which then moves to the nucleus, a similar process occurs after LPS stimulation but is less rapid. MAP3K7 signalling also occurs in the non-canonical NFκB pathway via NIK signalling (Ninomiya-Tsuji, Kishimoto *et al.* 1999). In these ways MAP3K7 can affect nuclear localisation of not only p50/RelA dimers but also RelB/p52 dimers.

It has been demonstrated in a mouse model that nuclear localisation of the p50/RelA NFκB dimers increases towards parturition and that blocking this nuclear localisation delays the onset of parturition (Lindstrom and Bennett 2005). Gene deletion of MAP3K7 has been shown to impair IKK and NFκB activity, therefore it is plausible that down regulation of MAP3K7 transcription, regulated by KCNMA1, could lead to a reduction in NFκB nuclear localisation and a delay in the onset of parturition.

MAP3K7 has also been shown to have a regulatory role in human tracheal smooth muscle proliferation, in these cells MAP3K7 promotes cell proliferation and suppresses contraction (Pera, Sami *et al.* 2011). As stated above a balance between hyperplasia and hypertrophy is critical for parturition to occur at the right juncture, an up-regulation of MAP3K7 transcription would lead to increased hyperplasia and subsequently decreased hypertrophy. Too little hypertrophy then means the cells do not receive the signals to produce the pro-contractile proteins and cytokines and therefore ultimately results in decreased contractility,

The ChIP-seq data and ChIP validation PCRs revealed KCNMA1 protein binding in the regulatory region of the MAP3K7 gene and so again this has revealed a further mechanism by which the KCNMA1 protein may have a role governing the timing of parturition.

8.7.4 The KCNMA1 Channel and KCNMA1 Protein Transcriptional Regulation

Interestingly the KCNMA1 channel has been shown to have a role in all the processes discussed above. Firstly, studies have revealed that potassium channels are responsible for hyperpolarising the plasma membrane, which is necessary for the cells to move from

G_1 to S (Wonderlin and Strobl 1996, Ouadid-Ahidouch and Ahidouch 2013). Secondly, KCNMA1 channels are sensitive to both voltage and calcium, transient increases in calcium can activate the KCNMA1 channel and in this way it can regulate calcium homeostasis. Finally, it has been shown that the KCNMA1 channel is activated by LPS and if this activation is blocked then the nuclear localisation of NFκB following LPS stimulation is abolished due to the non-degradation of IκBα (Dhulipala and Kotlikoff 1999, Papavlassopoulos, Stamme *et al.* 2006).

This ChIP-seq data adds to the picture of KCNMA1 regulation by demonstrating that the KCNMA1 channel does not regulate these processes purely in its role as a potassium channel, but that it may also have a role in regulating the expression of other proteins involved in these various processes.

8.8 Future Work

Further work needs to be undertaken to define the trigger which causes the switch in ion channel expression and splice variant profile; understanding this may enable this switch to be manipulated and in this way the timing of parturition could be delayed.

Further work examining the effect of different combinations of the various factors seen to be up-regulated at parturition (SP-A, MMPs, prostaglandins, TNF, IL-1β and IL6) on the expression and localisation of the KCNMA1, CACNA1C and CACNA1Gs is an important next step in order to better mimic the complexity of signalling that occurs at the onset of parturition.

Other areas which are also worthy of further investigation include clearly defining the link between the splice variants expressed and their corresponding protein isoforms, an examination of the phosphorylation of the KCNMA1 channel in order to better define the extent of the contribution of STREX-2 variant in the support of membrane depolarisation, determining the level of phosphorylation of the CACNA1C and CACNA1G to better understand how they are activated, defining the role of acetylation in the retention of the KCNMA1 channel and the membrane trafficking of the CACNA1Gs and optimisation of the ChIP protocols in order to define how expression of the KCNMA1, CACNA1C and CACNA1G is regulated.

Finally, the KCNMA1 protein transcription factor needs to be fully characterised including, defining what portion of the protein is required for its transcriptional activity, what stimulates its nuclear localisation, what genes it regulates and the nature of this regulation whether it is stimulatory or inhibitory.

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Appendices

Appendix 1 : The Single-Letter Amino Acid Code

G	Glycine	Gly	**P**	Proline	Pro
A	Alanine	Ala	**V**	Valine	Val
L	Leucine	Leu	**I**	Isoleucine	Ile
M	Methionine	Met	**C**	Cysteine	Cys
F	Phenylalanine	Phe	**Y**	Tyrosine	Tyr
W	Tryptophan	Trp	**H**	Histidine	His
K	Lysine	Lys	**R**	Arginine	Arg
Q	Glutamine	Gln	**N**	Asparagine	Asn
E	Glutamic Acid	Glu	**D**	Aspartic Acid	Asp
S	Serine	Ser	**T**	Threonine	Thr

Appendix 2 : Ethics

NHS
Health Research Authority

NRES Committee Yorkshire & The Humber - Bradford
Yorkshire & Humber REC Office
Millside
Mill Pond Lane
Meanwood
Leeds
LS6 4RA

Telephone: 0113 30 50128
Facsimile: 0113 85 56191

18 June 2012

Dr Neil Robert Chapman
Lecturer in Reproductive Medicine
University of Sheffield
Academic Unit of Reproductive and Developmental Medicine
Level 4, Jessop Wing
Tree Root Walk
Sheffield
S10 2SF

Dear Dr Chapman

Regulation of Human Myometrial Function by Transcription Factors, Ion Channels, Inflammatory Mediators and the Epigenome
REC reference: 12/YH/0229
Protocol number: STH 15863

Thank you for your letter of 18 June 2012, 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 Chair.

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, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior

to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to
the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

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

Document	*Version*	*Date*
Advertisement	1	18 April 2012
Covering Letter		
Evidence of insurance or indemnity		25 April 2012
Investigator CV		19 March 2012
Other: Mr Saurabh Gandhi CV		19 March 2012
Other: Student CV		19 April 2011
Other: Chapman et al 2004 JCEM paper demonstrating use of myometrial biopsies		
Other: Chapman et al 2005 Endocrinology paper demonstrating use		
Other: McElvy et al 2000 AJOG paper demonstrating safety of myometrial sampling used in this application and above studies		

Other: Letter seeking consent to use samples from a previous	2	18 June 2012
Participant Consent Form	3	18 June 2012
Participant Consent Form: Samples from previous study	2	18 June 2012
Participant Information Sheet	3	18 June 2012
Protocol	1	18 April 2012
REC application		20 April 2012
Response to Request for Further Information		18 June 2012
Summary/Synopsis		18 April 2012

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.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:
Notifying substantial amendments
Adding new sites and investigators
Notification of serious breaches of the protocol
Progress and safety reports
Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

12/YH/0229 **Please quote this number on all correspondence**

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

Yours sincerely

pp Dr Ian Woollands
Chair

Email: sinead.audsley@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Miss Angela Driscoll, Sheffield Teaching Hospitals NHS Foundation Trust*

Version 3 June 2012

The University Of Sheffield.

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

Study Number: REC Reference No. 12/YH/0229
Patient Identification Number for this study:

CONSENT FORM

A Study Looking at How the Womb Muscles Work During Pregnancy and Labour

Researchers:
Dr. Neil Chapman BSc, PhD, FHEA Lecturer in Reproductive Medicine

Mr. Saurabh Gandhi MD, MRCOG Honorary Senior Clinical Lecturer and Consultant Obstetrician and Sub-specialist in Feto-Maternal Medicine

Please read each statement carefully	Please initial each box
1) I confirm that I have read and understand the participant information sheet dated Version 3 June 2012 for the above study and have had the opportunity to ask questions.	
2) I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	
3) I confirm that I understand relevant sections of my medical records or data collected during the study may be looked at by individuals from the University of Sheffield, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	
4) I agree for my sample to be used in this study.	
5) I agree, where necessary, for my sample to be stored and used in future, ethically-approved research looking at how the womb muscles work in pregnancy and labour.	
6) I agree to take part in the above study.	

Name of Participant	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Version 3 June 2012

The University Of Sheffield.

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

Participant Information Sheet

A Study Looking at How the Womb Muscles Work During Pregnancy and Labour

You are being invited to take part 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 friends, relatives and your GP 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.

1) What is the purpose of the study?

We do not understand how the muscles of the womb stay relaxed during pregnancy, and then begin to contract at the end of pregnancy to lead to the birth of the baby. We also do no fully know why contractions of the womb muscles sometimes start too soon and lead to premature birth. Understanding these processes may help us develop treatments to prevent premature birth. Using small pieces of the muscle from the womb, we are carrying out research to find out how the womb muscle grows during pregnancy, and how it works when labour starts. In the laboratory we study muscle tissue from women whose pregnancies lasted the full nine months, and from those having a premature birth. These tissues are only obtained if women are having a Caesarean section either after some time in labour or when they have not been in labour.

2) What will be involved if I agree to take part in the study?

We are approaching all pregnant women in case they eventually need to have a Caesarean section to deliver their baby. Roughly one in five pregnancies is delivered by a Caesarean at the Jessop Wing. If you agree to take part in the study, and require a Caesarean section to deliver your baby, we will plan to take one small sample of muscle from your womb. The small sample of muscle from the inner lining of the womb will be taken towards the end of your operation, after the baby has been safely delivered and the placenta removed. We must emphasize that taking this sample only takes an additional one-two minutes and does not in any way pose any risks to yourself or your baby. We will then examine these muscle cells to see how they control the formation of special proteins and other molecules which play a role in how the womb muscle grows and works during pregnancy and labour as detailed in section (1) above.

3) When and where will the samples be taken?

The samples will be taken in the operating theatre when you are having your Caesarean section after the baby and the placenta have been delivered.

4) What will happen to the sample after donation?

Essentially, the biopsy sample will be used by the research team in one of two ways. Firstly, it can be used to isolate the actual muscle cells that make the uterus contract. Once we have isolated such cells, we can then grow them in the lab to see what chemicals in the body cause the cells to contract and how the cell's genes regulate this process. Importantly, we can then also try and find out which chemicals stop these muscle cells from contracting to early.

Secondly, we may store part of the biopsy in deep freeze. This means that we can, at a later date in the study, extract many of the chemicals from the biopsy and compare how these change between women who are pregnant but not in labour with those who are in active labour. These samples will be used to make sure our research findings from the cells we grow are real. In some cases, if you decide to participate in this study, the sample donated may be stored for use at a later date in similar, ethically-approved research looking at how the womb muscles work in pregnancy and labour.

5) Are there any risks to me or my baby from taking part in this study?

No. There are no risks additional to those of your Caesarean section from taking this sample from you, or from any other aspect of this research study. It will not significantly increase your operating time, and takes no more than an extra one-two minutes. The taking of biopsies will not cause any additional discomfort nor affect your recovery.

Whilst there is no information on whether future fertility would be affected by taking part in this study, we believe this would be unlikely because the small section of the womb muscle taken for the biopsy would be replaced after birth as part of the natural healing process occurring in the womb.

6) Do I have to take part in the study?

No. You are free to refuse to join the study. If you do join and change your mind, you are free to withdraw at any time. This will in no way affect how you are looked after during your
stay in hospital.

7) What other information will be collected in the study?

No other information will be collected from this study. With your permission we would like to be able to look for other related substances in the tissue obtained that may explain how the womb works during pregnancy and child birth as detailed in section (1) above.

8) Will the information obtained in the study be confidential?

Yes. We will follow all ethical and legal practise to keep your personal information confidential. The greatest care will also be taken to ensure your identity is not revealed to anyone outside the research team. Anything you say to any member of the research team will also be treated in the strictest confidence and no names will be mentioned in any reports of the study. Care will be taken so that individuals cannot be identified from details in reports of the results of the study.

9) Will anyone else be told about my participation in the study?

As this will not affect your clinical treatment we will not be informing anyone of your involvement in the study. However we can let your family doctor know should you specifically request us to do so.

10) What if I wish to complain about the way in which this study has been conducted?

If you have any cause to complain about any aspect of the way in which you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you and are not compromised in any way because you have taken part in a research study. If you have any complaints or concerns please contact the project coordinator: Dr Neil Chapman on 0114 2268530. Otherwise you can use the normal hospital complaints procedure and contact the following person: Mrs. Janet Wilson, Complaints Manager, Royal Hallamshire Hospital, G Floor, Room 39A, Glossop Road, Sheffield S10 2JF. In the alternative you could write to the Director of Research , Sheffield Teaching Hospitals Research Unit, 305 Western Bank Sheffield S10 2TJ. Otherwise you can use the normal University complaints procedure and send your complaint in writing addressed to "The Registrar and Secretary, University of Sheffield, Firth Court, Western Bank, Sheffield, S10 2TN".

The University Of Sheffield.

Department Of Human Metabolism.

Patient's name and Address placed here		**Dr. Neil Chapman, Non-Clinical Lecturer** **Ms. Val Aram, Secretary** Department of Human Metabolism Academic Unit of Reproductive & Developmental Medicine, Level 4, Jessop Wing, Tree Root Walk, Sheffield S10 2SF Telephone: 00 44 (0) 114 226 8530/2268536 Fax: 00 44 (0)114 226 8538 Email: n.r.chapman@sheffield.ac.uk

Date 2012

Dear {patient's name},

Re: Consent to Use Previously Donated Womb Sample in a new Ethically-approved Study entitled:
A Study Looking at How the Womb Muscles Work During Pregnancy and Labour **Ethics**
REC Ref. No. 12/YH/0229

In {month and Year} you underwent an elective Caesarean section to deliver your baby. At that time, you also gave consent for my research team to obtain a small piece of the muscle of your womb to allow us to study how the uterus works during pregnancy and labour. Once the biopsy was taken it was divided into two sections; one was used immediately to isolate muscle cells; the remainder frozen and stored to be used at a later point in the study. While that aspect of the study has now recently ended, we still have the frozen section of your donated biopsy in storage.

As such, I am now writing to you to ask your permission and consent to use that frozen biopsy in a future ethically-approved study. This new study is essentially an extension of the work you participated in previously and the biopsy you donated can still be used in this work because it has been frozen. We will still be looking at the molecules that regulate how the uterus works during pregnancy and labour. You are, of course, also able to decline this request without prejudice whatsoever. If this is the case, the biopsy will be disposed of in accordance with the Human Tissue Act (2004).

I have enclosed the Participant Information Sheet, Version 2 June 2012, for the new ethically- approved study. Please read this before making your decision as it gives details of how your frozen biopsy will be used in the new study.

I have also enclosed a consent form for you to read carefully. On that form you have two options; essentially:

1) You give permission and consent for the stored biopsy to be used in the future study.
2) You do not give permission and request the biopsy be disposed of in accordance with the Human Tissue Act (2004).

1

Once you have read the information sheet, I would be very grateful if you can tick only one of the options that apply to you, sign and date the form and return it to me in the pre-paid, self- addressed envelope provided. I can then process the biopsy according to your wishes.

If you have any questions at all regarding this matter, please do not hesitate to contact me on the above telephone number or email address and I will be happy to discuss them with you.

Finally, may I take this opportunity to thank you for participating in the original study; it really is very much appreciated.

With best wishes,
Yours sincerely,

Dr. Neil Chapman, B.Sc. (HONS.), Ph.D., P.G.Cert.H.E., F.H.E.A. Study
Chief Investigator

Version 2 June 2012

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

The University Of Sheffield.

Study Number: REC Ref. No. 12/YH/0229

CONSENT FORM

A Study Looking at How the Womb Muscles Work During Pregnancy and Labour

Researchers:
Dr. Neil Chapman BSc, PhD, FHEA Lecturer in Reproductive Medicine

Mr. Saurabh Gandhi MD, MRCOG Honorary Senior Clinical Lecturer and Consultant Obstetrician and
Sub-specialist in Feto-Maternal Medicine

Please read each statement carefully	Please initial only
1) I have read the participant information sheet Version 3 June 2012 and give permission and consent for my stored biopsy to be used in the ethically approved research study listed above.	
2) I do not give permission and request my donated biopsy be disposed of in accordance with the Human Tissue Act (2004).	

Name of Participant Date Signature

Appendix 3 : Dot Blots

Dot Blots

Dot blots were utilised to optimise the protein and antibody concentrations for use in western blotting. A dot blot is a simplified version of a western blot where the proteins are not first separated by electrophoresis. It is a quick and effective method of determining the optimum combination of protein amount and primary antibody dilution. In this study, dot blots were used to determine the following values: amount of protein to load on the gel, what concentration of primary antibody to use, what temperature the primary antibody should be incubated at and which detection reagent to use to develop the blots was determined from these dot blots.

Different quantities of protein (5, 10 and 50μg) were applied directly to nitrocellulose membrane (Geneflow, Staffs) as dots, then incubated with different dilutions of primary antibody. The only exceptions to these optimisations are the amount of protein loaded from the soluble nuclear and chromatin bound nuclear fractions. These fractions do not contain enough protein to load 5μg per gel and so instead an equal volume of these fractions from each sample was loaded on the gel. This meant these fractions were only used once to determine the presence or absence of a particular protein and not the relative quantities of the protein present as a result of the different stimulations. Secondary antibody was then added and the blots developed using Westar Supernova detection reagents.

Clear dots were produced for each of the antibodies (Figure 4.3.1). The CACNA1G (Alomone, Jerusalem), KCNMA1 C-Terminal (Alomone, Jerusalem) and CACNA1C C-Terminal (Abcam, Cambridge USA) quickly became over exposed and so these dot blots were repeated using the lowest antibody dilution, with incubations at either room temp or 4ºC and developed using the less sensitive EZ-ECL detection kit (Geneflow, Staffs), with these combinations all three produced clear dots (Figure 4.3.1)

The KCNMA1 C-terminal (Alomone, Jerusalem) had a high level of background and so the primary antibody was diluted in blocking buffer and incubated at 4ºC overnight, this significantly reduced the level of background.

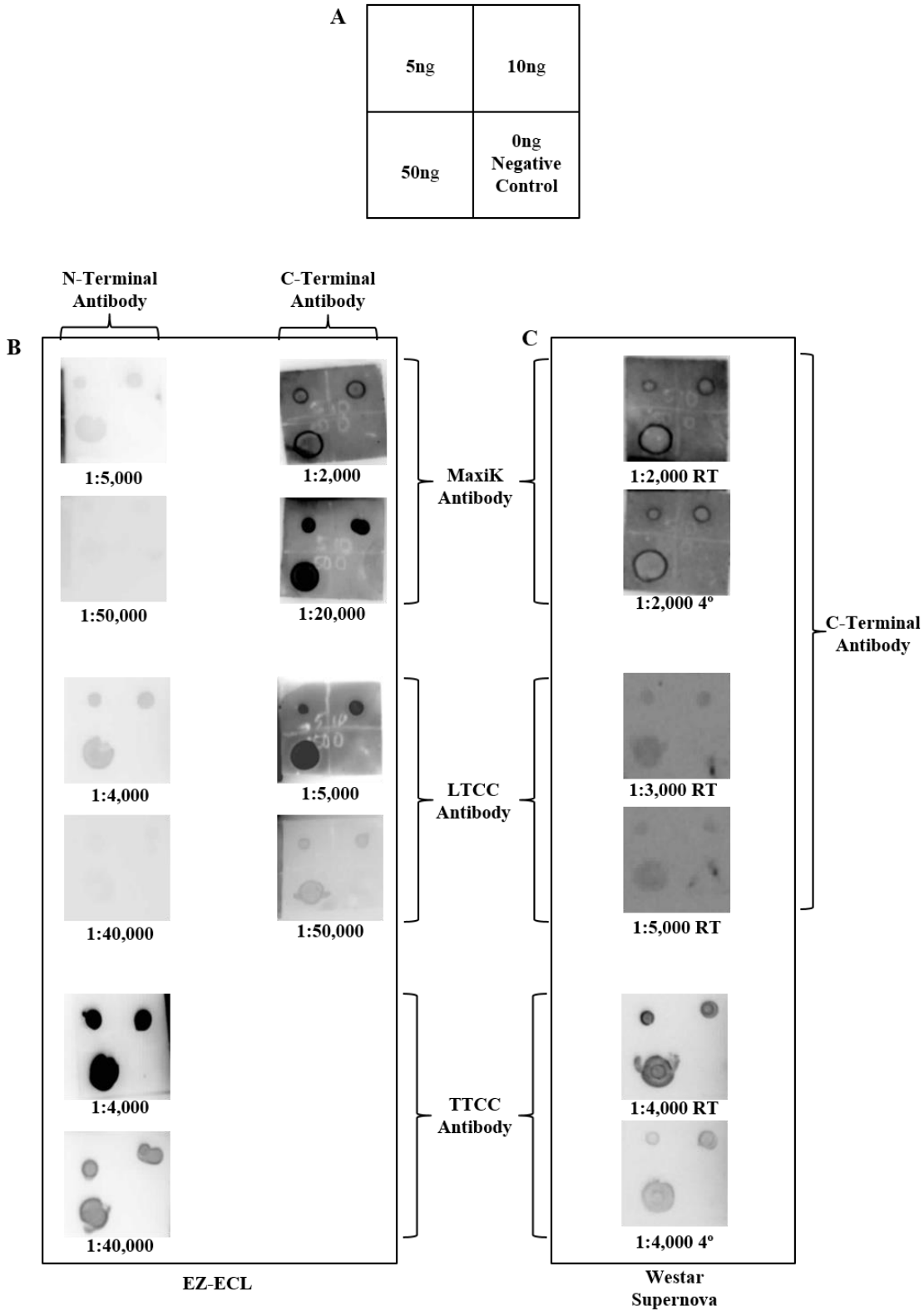

Figure 4.3.1 Optimisation of Protein and Antibody concentrations using Dot Blot The amount and location of the protein blotted on to each membrane is illustrated (A) The lack of dot in the negative control of each blot and the lack of background on the majority of blots show the block is effective and the antibody is specific. The dark spots indicate the antibody is detecting protein. Blots were developed with either WestStar Supernova (B) or EZ-ECL (C) detection reagents. Dilutions of primary antibodies used are detailed below the individual blot