Role of Internal Calcium Stores in the KCA2.3 Regulation of Human Uterine Smooth Muscle Contractions

Bri Kestler
University of South Alabama, bek1522@jagmail.southalabama.edu

Follow this and additional works at: https://jagworks.southalabama.edu/theses_diss

Part of the Cell Biology Commons

Recommended Citation
Kestler, Bri, "Role of Internal Calcium Stores in the KCA2.3 Regulation of Human Uterine Smooth Muscle Contractions" (2021). Theses and Dissertations. 9.
https://jagworks.southalabama.edu/theses_diss/9

This Dissertation is brought to you for free and open access by the Graduate School at JagWorks@USA. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of JagWorks@USA. For more information, please contact mduffy@southalabama.edu.
ROLE OF INTERNAL CALCIUM STORES IN THE KCA2.3 REGULATION OF HUMAN UTERINE SMOOTH MUSCLE CONTRACTIONS

A Dissertation

Submitted to the Graduate Faculty of the University of South Alabama in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Basic Medical Sciences
Physiology and Cell Biology

by
Bri Kestler
B.S., Biochemistry, 2003
M.M.S., Physician Assistant Studies, 2010
December 2021
“Family is a life jacket in the stormy sea of life”

I would not have completed this journey without my family. I will forever be thankful to my husband. He understood this was a goal of mine and never once faltered from his promise to help me get through it. Sometimes he talked me down and other times he rallied me up; he truly is an extraordinary husband and father. This work is dedicated to my mother who came to my rescue emotionally, mentally, and physically on many occasions. And to my daughter, who doesn’t realize how much she pushed me to complete my dreams, if only to be a strong role model for her.

And to all the preemies.
They say it takes a village to raise a child, but I would say that statement holds true with graduating a predoc. I would like to express a heartfelt thank you to my committee members, Drs. David Weber, Jonathan Scammell, Mary Townsley, Abu-Bakr Al-Mehdi, and Brian Fouty, who guided me through the infant stages of my project to the point where it is today. Dr. Mark Taylor deserves his own special thank you for allowing me to figure out my own course, as disorderly as it was at times, and reach the end in a manner I am proud of. I appreciate my colleagues in the University of South Alabama Physician Assistant Program for their undying support and coffee runs. Thank you to Kelli Roberson, Judi Naylor, and Jenn Collins for daily encouragement and safeguarding my lab supplies. My gratitude to Dr. Chung-sik Choi who kept me company in the lab and taught me his craft.

This project would not have been possible without the assistance of the University of South Alabama OB/GYN Faculty and Research Staff. I am indebted to Drs. Mimi Munn and Tracy Roth for sharing their patients with me, Dr. Amy Hewes for being the go between with the residents, having the perfect kind of OR music playlist, and dealing with my text messages, and Catera Duhon who did whatever she could to make this project happen. Thank you.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xi</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>xiv</td>
</tr>
<tr>
<td>CHAPTER I INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>CHAPTER II BACKGROUND</td>
<td>4</td>
</tr>
<tr>
<td>Uterine anatomy</td>
<td>4</td>
</tr>
<tr>
<td>Hormonal regulation</td>
<td>6</td>
</tr>
<tr>
<td>Progesterone</td>
<td>6</td>
</tr>
<tr>
<td>Estrogen</td>
<td>8</td>
</tr>
<tr>
<td>Myometrial contraction physiology</td>
<td>10</td>
</tr>
<tr>
<td>Myometrial excitation</td>
<td>10</td>
</tr>
<tr>
<td>Myometrial force generation</td>
<td>13</td>
</tr>
<tr>
<td>Potassium channels</td>
<td>14</td>
</tr>
<tr>
<td>Small-conductance calcium-activated potassium channels</td>
<td>17</td>
</tr>
<tr>
<td>Modulation of SKCa channels</td>
<td>21</td>
</tr>
<tr>
<td>Internal calcium store channels</td>
<td>22</td>
</tr>
<tr>
<td>Agents that increase IP3 generation</td>
<td>25</td>
</tr>
<tr>
<td>Topic</td>
<td>Page</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>25</td>
</tr>
<tr>
<td>Prostaglandins</td>
<td>27</td>
</tr>
<tr>
<td>KCa channels and internal calcium store channels</td>
<td>28</td>
</tr>
<tr>
<td>Preterm labor</td>
<td>30</td>
</tr>
<tr>
<td>Management of preterm labor</td>
<td>32</td>
</tr>
<tr>
<td>Tocolytic agents</td>
<td>33</td>
</tr>
<tr>
<td>CHAPTER III - MATERIALS AND METHODS</td>
<td>35</td>
</tr>
<tr>
<td>General materials and methods</td>
<td>35</td>
</tr>
<tr>
<td>Patient selection</td>
<td>35</td>
</tr>
<tr>
<td>Human tissue samples</td>
<td>35</td>
</tr>
<tr>
<td>Materials</td>
<td>37</td>
</tr>
<tr>
<td>Immunofluorescent microscopy</td>
<td>39</td>
</tr>
<tr>
<td>Tissue myography</td>
<td>40</td>
</tr>
<tr>
<td>Uterine ring preparations</td>
<td>40</td>
</tr>
<tr>
<td>Myographical recordings</td>
<td>40</td>
</tr>
<tr>
<td>Drugs</td>
<td>41</td>
</tr>
<tr>
<td>Statistical analysis</td>
<td>41</td>
</tr>
<tr>
<td>CHAPTER IV – RESULTS</td>
<td>43</td>
</tr>
<tr>
<td>Evaluate the expression and distribution of KCa2.3 and IP3R channels</td>
<td>43</td>
</tr>
<tr>
<td>in human myometrium</td>
<td></td>
</tr>
<tr>
<td>Characterize spontaneous and augmented contractions of human uterine</td>
<td>47</td>
</tr>
<tr>
<td>tissue</td>
<td></td>
</tr>
<tr>
<td>Reproductive state and spontaneous contraction characteristics</td>
<td>47</td>
</tr>
<tr>
<td>Functional impact of CyPPA on human uterine smooth muscle contractions</td>
<td>54</td>
</tr>
<tr>
<td>Assess the role of internal calcium stores in KCa2.3 modulation of</td>
<td>61</td>
</tr>
<tr>
<td>human uterine contractility</td>
<td></td>
</tr>
<tr>
<td>Examine CyPPA attenuation of uterine contractions with oxytocin</td>
<td>61</td>
</tr>
<tr>
<td>treatment</td>
<td></td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Demographic and clinical data of non-pregnant women</td>
<td>36</td>
</tr>
<tr>
<td>2. Demographic and clinical data of pregnant women</td>
<td>37</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Anatomical regions and layers of the human uterus</td>
</tr>
<tr>
<td>2.</td>
<td>Diagram of uterine smooth muscle contractile mechanisms</td>
</tr>
<tr>
<td>3.</td>
<td>Diagram of SKCa channels with associated calmodulin and regulatory proteins, CK2 and PP2A</td>
</tr>
<tr>
<td>4.</td>
<td>Calcium-induced calcium release from IP3R channels</td>
</tr>
<tr>
<td>5.</td>
<td>GPCR mechanism of IP3 generation</td>
</tr>
<tr>
<td>6.</td>
<td>CyPPA suppression of spontaneous and oxytocin-induced contractions of isolated mouse uterine strips</td>
</tr>
<tr>
<td>7.</td>
<td>Dissection and allocation of human uterine tissue</td>
</tr>
<tr>
<td>8.</td>
<td>KCa2.3 and IP3R channels are present in non-pregnant human myometrium</td>
</tr>
<tr>
<td>9.</td>
<td>Varying staining patterns of KCa2.3 and IP3R channels are visualized in non-pregnant tissue</td>
</tr>
<tr>
<td>10.</td>
<td>KCa2.3 and IP3R channels are present in pregnant human myometrium</td>
</tr>
<tr>
<td>11.</td>
<td>Expression and distribution patterns of KCa2.3 and IP3R are not consistent in pregnant tissue</td>
</tr>
<tr>
<td>12.</td>
<td>Frequencies of human uterine contractions in non-pregnant and pregnant tissue</td>
</tr>
<tr>
<td>13.</td>
<td>Amplitudes of human uterine contractions in non-pregnant and pregnant tissue</td>
</tr>
<tr>
<td>14.</td>
<td>Histogram of spontaneous human uterine contraction frequencies</td>
</tr>
<tr>
<td>15.</td>
<td>Histogram of spontaneous human uterine contraction amplitudes</td>
</tr>
</tbody>
</table>
16. Comparison of frequencies and amplitude between non-pregnant and pregnant samples ................................................................. 52
17. Variation of contraction frequency and amplitude within same subject samples ...... 53
18. Effect of CyPPA on human uterine contractions in concentration-response studies .................................................................................. 55
19. Effect of increasing CyPPA on human uterine contraction frequency of individual samples .................................................................. 56
20. Effect of increasing CyPPA on human uterine contraction amplitude of individual samples .................................................................. 56
21. Summary of increasing CyPPA concentration on contraction frequency ........... 57
22. Summary of increasing CyPPA concentration on contraction amplitude ............ 58
23. Effect of increasing CyPPA on human uterine contraction AUC of individual samples ........................................................................... 59
24. Summary of increasing CyPPA concentration on contraction AUC ..................... 60
25. Comparison of non-pregnant and pregnant human myometrial samples after treatment with 30 µM of CyPPA .................................................. 62
26. Effect of CyPPA pretreatment protocol on pregnant human uterine contraction frequency, amplitude, and AUC of individual samples ................. 64
27. Effects of CyPPA and OT treatment on human myometrial contractility ............. 65
28. Effect of OT pretreatment protocol on pregnant human uterine contraction frequency, amplitude, and AUC of individual samples ........................................ 67
29. Effects of OT and CyPPA treatment on human myometrial contractility ............. 68
30. Difference in AUC of contractions between two treatment protocol involving varying concentrations and application order of CyPPA and OT ........ 69
31. Myographical recording of changes to contractions during execution of the CPA and CyPPA protocol ................................................................. 70
32. Summary of contractile results in the comparison of increasing CyPPA concentrations with or without CPA pretreatment ................................. 73
33. Alterations to spontaneous contractions of pregnant uterine tissue after administration of GPCR or PLC agents .............................................................. 75

34. Myographical recordings of pregnant tissue samples from the same subject incubated with CyPPA prior to administration of GPCR or PLC agents .............. 76

35. Effects of oxytocin on pregnant uterine contractions in presence of KCa2.3 channel modulators .................................................................................. 77

36. Effects of prostaglandin F2a on pregnant uterine contractions in presence of KCa2.3 channel modulators ............................................................................ 78

37. Effects of m-3M3FBS on pregnant uterine contractions in presence of KCa2.3 channel modulators .................................................................................. 79

38. Effects of U73122 on pregnant uterine contractions in presence of KCa2.3 channel modulators ...................................................................................... 81

39. Myographical recordings of pregnant tissue incubated with apamin prior to administration of GPCR and PLC agents ........................................................................... 82

Appendix figures
40. Secondary antibody only controls ........................................................................ 126

41. Concentration-response curves for oxytocin, PGF2α, m-3M3FBS, and U73122 .................................................................................................................. 127
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>adenyl cyclase</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>β&lt;sub&gt;2&lt;/sub&gt;AR</td>
<td>beta-adrenergic receptor</td>
</tr>
<tr>
<td>BKCa</td>
<td>large-conductance calcium-activated potassium channel</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>CaMBD</td>
<td>calmodulin-binding domain</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAP</td>
<td>contraction associated protein</td>
</tr>
<tr>
<td>CICR</td>
<td>calcium induced calcium release</td>
</tr>
<tr>
<td>CK2</td>
<td>casein kinase 2</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>CPA</td>
<td>cyclopiazonic acid</td>
</tr>
<tr>
<td>Cx43</td>
<td>connexin-43</td>
</tr>
<tr>
<td>CyPPA</td>
<td>cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]amine</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptors</td>
</tr>
<tr>
<td>ERE</td>
<td>estrogen response elements</td>
</tr>
</tbody>
</table>
FP
PGF2α receptor

GPCR
G protein-coupled receptor

IBC
IP3-binding core

IL-11
interleukin 11

IP3R
inositol 1,4,5-triphosphate receptor

K_{ATP}
ATP-sensitive potassium channels

KCa
calcium-activated potassium channels

KCa2.3
small conductance calcium-activated potassium channels

K_{V}
voltage-gated potassium channel

MLCK
myosin light chain kinase

MLCP
myosin light chain phosphatase

MSMC
myometrial smooth muscle cells

NALCN
Na^{+}-activated leak channel, non-selective channel

NCX
sodium calcium exchanger

NF-κB
nuclear factor kappa B

nPR
nuclear progesterone receptor

NSCC
nonselective cation channels

OT
oxytocin

OTR
oxytocin receptor

PAECs
pulmonary arterial endothelial cells

PG
prostaglandin

PGF2α
prostaglandin-F2α

PIP2
phosphatidylinositol 4,5-bisphosphate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKA</td>
<td>protein-kinase-A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase type C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PLCβ</td>
<td>phospholipase C-β</td>
</tr>
<tr>
<td>PMCA</td>
<td>plasma membrane calcium-ATPase</td>
</tr>
<tr>
<td>PMVECs</td>
<td>pulmonary microvascular endothelial cells</td>
</tr>
<tr>
<td>PP2A</td>
<td>protein phosphatase 2A</td>
</tr>
<tr>
<td>PPROM</td>
<td>preterm premature rupture of membranes</td>
</tr>
<tr>
<td>PTB</td>
<td>preterm birth</td>
</tr>
<tr>
<td>RyR</td>
<td>ryanodine receptor channel</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarcoplasmic reticulum calcium ATPase</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TRPV4</td>
<td>transient receptor potential subtype-4</td>
</tr>
<tr>
<td>VGCC</td>
<td>voltage-gated calcium channel</td>
</tr>
</tbody>
</table>
ABSTRACT

Kestler, Bri, Ph.D., University of South Alabama, December 2021. Role of internal calcium stores in the KCa2.3 regulation of human uterine smooth muscle contractions. Chair of Committee: Mark S. Taylor, Ph.D.

Minute-to-minute regulation of intercellular calcium concentrations is essential to allow the uterus to phasically contract and relax in a manner required for delivery. A specific class of calcium-activated potassium (KCa2.3) channels has been strongly implicated in the negative feedback control of intracellular calcium levels. Overexpression of KCa2.3 channels compromises labor by diminishing uterine contractions. Pharmacologic positive modulation of KCa2.3 channels with the small molecule cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]amine (CyPPA), increases the calcium sensitivity of KCa2.3 channels and promotes membrane hyperpolarization through potassium efflux. Local coupling of plasma membrane channels, such as TRP and KCa, and internal store release channels is required for activation and regulates tone in multiple vessels. A similar relationship in human uterine tissue is proposed based on preliminary studies suggesting internal calcium stores play a role in KCa-dependent feedback. This study characterized spontaneous and CyPPA augmented contractions and the role of internal stores in the KCa feedback control of human myometrial contractions.
Dual staining immunofluorescence allowed us to observe the location and distribution of KCa2.3 and Ip3R channels in human myometrium. We found multiple staining patterns of KCa2.3 channels, with a general shift from more punctated signal in the non-pregnant tissue to that of diffuse staining with varying intensity within pregnant tissue. IP3R signal in human myometrium was generally diffuse with specific areas of clustering and was generally preserved between gestational states. Proximity of KCa2.33 and IP3Rs differed between samples with some having very distinct individual channel signal and others showing convergence of signal, possibly due to colocalization.

Using isometric force myography, we found that spontaneous phasic contractions of non-pregnant and pregnant human myometrial tissue varied considerably and were significantly different between gestational groups. CyPPA suppressed contractions in both gestational groups and was more effective at reducing amplitude in pregnant samples. CyPPA reduced contractility in tissue depleted of internal calcium stores via cyclopiazonic acid; indicating the relaxant effects of CyPPA do not rely on internal calcium store release in an unstimulated environment. CyPPA was able to significantly reduce contractility in the presence of oxytocin and had the ability to blunt oxytocin’s pro-contractile mechanisms further supporting its potential as a tocolytic agent.

In all aspects of the study there was variability in response to agents proposing functional architecture determines the specificity of tissue response. The inability of oxytocin to increase contractility when CyPPA was present suggests the cellular arrangement between internal store release channels and KCa2.3 play a role in the negative feedback control of uterine contraction and offer new insights into the innate variability of human uterine contractions.
CHAPTER 1
INTRODUCTION

The United States has the highest rates of preterm birth in the industrial world. Preterm birth is defined as delivery before 37 weeks gestation and is the leading cause of newborn death.\textsuperscript{1-4} The annual cost from disability and number of years lost due to ill health in preterm neonates is estimated at $26.2 billion a year in the United States.\textsuperscript{2} While the use of medications to combat preterm labor, or tocolytic medications, are not directly associated with a reduction in neonatal complications, the brief delay of birth allows for administration of antenatal corticosteroids.\textsuperscript{5,6} Application of antenatal corticosteroids decreases fetal complications such as respiratory distress syndrome, intraventricular hemorrhage, and necrotizing enterocolitis.\textsuperscript{3,6} Over the past decade, survival rates have improved for preterm neonates, but this has been due to advances in neonatal care rather than improvements in prenatal care.\textsuperscript{3,4} Spontaneous preterm labor accounts for approximately 70\% of preterm births, with the remaining 30\% accounting for medically induced birth due to maternal or fetal complications.\textsuperscript{2} With one in ten children born prematurely, and only ineffective and outdated medications available for treatment, regulatory mechanisms of uterine contractions need to be studied for identification of new pharmacologic targets.
Effective uterine contractions require an increase in cytosolic calcium concentration, with depolarization of uterine smooth muscle occurring via calcium entry through voltage-gated calcium channels (VGCC). An efflux of potassium ions through calcium-activated potassium channels (KCa) results in membrane potential hyperpolarization, reduced cytosolic calcium, and smooth muscle relaxation. A specific class of small conductance KCa channels (KCa2.3) has been strongly implicated in this negative feedback control of uterine contractions. Overexpression of KCa2.3 channels impedes uterine contractions through increased hyperpolarizing effects and closure of VGCCs. Exploitation of KCa2.3 channels has surfaced as a new hope for tocolytic therapy. Studies suggest enhancing calcium sensitivity of KCa2.3 channels with the compound cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]amine (CyPPA) reduces the force of uterine contractions by amplifying calcium-dependent feedback. Indeed, CyPPA treatment delays delivery in preterm laboring mice.

Calcium release from the sarcoplasmic reticulum (SR) leads to increased intracellular calcium levels and muscle contraction. Preliminary studies suggest internal store calcium release may be crucial for CyPPA suppression of uterine contractions through KCa2.3 channel positive modulation. A role for internal calcium stores may be similar to that described recently in vascular endothelium where close proximity between inositol 1,4,5-triphosphate receptors (IP3Rs) and KCa2.3 channels provides an intrinsic, tunable mechanism for calcium feedback. Uterine contractility may be altered due to an increase in local calcium concentrations around KCa2.3 channels which intensifies hyperpolarization. A functional relationship could explain data
from our laboratory pointing to a synergistic effect between IP3R agonists and CyPPA. Assessing the link between internal calcium stores and KCa2.3 channels in human uterine tissue would open new targets and strategies for tocolytic therapy. Therefore, this proposal tests the **HYPOTHESIS** that internal calcium stores drive KCa2.3 feedback suppression of human myometrial contractility.

**AIM 1**: Evaluate the expression and distribution of KCa2.3 and IP3R channels in human myometrium.

**AIM 2**: Characterize spontaneous and augmented contractions of human uterine tissue.

**AIM 3**: Assess the role of the internal calcium stores in KCa2.3 modulation of human uterine contractility.
CHAPTER II
BACKGROUND

Uterine anatomy

The most superior aspect of the uterus is the fundus. Moving inferior, next is the corpus, followed by the lower uterine segment and the isthmus, ending with the cervix. The human uterus consists of three layers: an outer covering, the serosa; a middle muscular layer, the myometrium; and an inner mucous membrane, the endometrium. The serosal layer is a continuation of peritoneal epithelium and encloses almost the entire uterus. The endometrium is a simple mucous membrane that is regulated by hormonal secretion and is the site of fetal tissue implantation. The myometrium is the thickest layer of the uterus and undergoes the most pregnancy associated changes. Within the myometrium are an outer longitudinal muscle layer and an inner circular layer. The muscle fiber bundles of the longitudinal muscle layer are oriented along the long axis of the uterus, with individual myometrial smooth muscle cells (MSMCs) arranged in the long axis of the bundle itself. The muscle cells of the circular layer are more diffusely bundled, but generally arranged concentrically around the longitudinal axis of the uterus. In humans, there is not clear delineation between muscle layers as there is in other animal species.
Figure 1. **Anatomical regions and layers of the human uterus.** The serosa is the outer layer of the uterus that is continuous with the peritoneal epithelium. The myometrium is the middle muscular layer composed of longitudinal and circular muscle fibers. The endometrium is the inner mucosal membrane that includes the decidua during pregnancy.
Uterine myocytes are spindle-shaped cells with a predominante nucleus, well-developed sarcoplasmic reticulum (SR), ample caveolae, and gap junctions between neighboring cells. Myometrial growth during pregnancy is accomplished by an increase in the number of MSMCs (hyperplasia), and to a lesser degree an increase in the size of cells (hypertrophy). Myocytes increase from 2 – 20 microns in diameter and 50 – 1,000 microns in length during gestation. MSMCs are densely packed with myofilaments and dense bodies, which together make up 80-90% of the cell. MSMC myofilaments are either thin filaments 6-8 nm in diameter and composed mainly of globular actin, or thick filaments 15-18 nm in diameter composed of myosin. There are six times more actin containing filaments than myosin filaments in the myometrium. Actin filaments are anchored to the cell and the cytoskeleton via dense bodies and dense bands. Dense bodies are in the cytosol and bridge actin thin filaments together along the contractile plane. They serve as anchors for actin filaments to exert force onto and bring polar cell membranes together. Dense bands are associated with the peripheral membrane and form structures around the circumference of the cell, allowing actin filaments to be tethered to the cytoskeleton. Dense bands allow forces to be transmitted toward the peripheral membrane during generation of mechanical tension.

**Hormonal regulation**

**Progesterone**

While pregnancy is a complicated process that is still not fully understood it is known to be partly controlled by two steroid hormones, progesterone and estrogen, and
their metabolites. Progesterone is produced by the adrenal cortex and the ovaries during a non-pregnant state, with a transition to the ovarian corpus luteum and the placenta during pregnancy. Progesterone promotes a pro-pregnancy uterine phenotype mainly through binding of progesterone to a nuclear progesterone receptor (nPR) that functions as an intracellular ligand-activated transcription factor to mediate gene expression. The human PR gene encodes for two isoforms: the full length PR-B and the truncated PR-A. PR-B is the predominate isoform during early pregnancy and the largest mediator of progesterone-induced transcriptional activity. The main genomic mechanism in which PR-B promotes relaxation is through the suppression of contraction associated protein (CAP) gene expression. Suppressed CAPs include uterotonic receptors, such as the oxytocin (OT) receptor (OTR) and prostaglandin-F2a (PGF2a) receptor (FP), allowing progesterone to diminish responsiveness to gestational hormones. Another is the gap junction protein connexin-43 (Cx43), which unsuppressed allows for the propagation of action potentials and direct intercellular communication between myometrial cells. PR-B inhibits myometrial inflammation and desensitizes myometrial tissue to inflammatory stimuli through suppression of IL-11 expression, impairment of the NF-kB inflammatory pathway through upregulation of NF-kB inhibitors, and inhibition of TNF- and thrombin-induced weakening of the amnion membrane. PR-B also stimulates myometrial relaxation by augmenting the cAMP/protein-kinase-A (PKA) signaling pathway. Increased cAMP/PKA inhibits phospholipase C (PLC) from elevating cytosolic Ca\(^{2+}\) levels, via stimulation by uterotonins, such as OT and PGF2a.

In most species labor is triggered at term by a systemic decrease in progesterone, though in humans’ progesterone levels remain elevated up until the final phase of labor.
One of the possible mechanisms for this functional progesterone withdrawal includes a switch in the relative levels of PR isoforms.\textsuperscript{38,44} During early pregnancy, PR-B dominates with PR-A levels being much lower creating a PR-A:PR-B ratio of approximately 1:2.\textsuperscript{38} Myometrial PR-A levels increase late in pregnancy causing a transition to PR-A dominance and causing the PR-A:PR-B ratio to be 3:1 at time of delivery.\textsuperscript{38} PR-A is a repressor of PR-B, especially when its levels dominate.\textsuperscript{38,44} PR-A promotes inflammation by inhibiting the PR-B upregulation of NF-kB inhibitors, thereby increasing expression of proinflammatory genes (e.g., PTGS2, IL8, IL1A, and PTX3).\textsuperscript{44} These findings support the idea that progesterone promotes a relaxed myometrial state for most of pregnancy via PR-B mediated genomic actions, which are inhibited late in pregnancy with an increased expression of the PR-A isoform. Progesterone has also been shown to rapidly activate intracellular signaling pathways through progestin membrane receptors. Progestin membrane receptors activate inhibitory G proteins resulting in reduced cAMP production and inhibition of adenyl cyclase activity, as well activation of p38 mitogen-activated protein kinase which causes phosphorylation of myosin light chain protein.\textsuperscript{48,49} These findings suggest that progesterone could promote myometrial contractions during labor through membrane receptor signaling. There is also evidence supporting progesterone’s ability to trigger calcium release from internal stores through progestin membrane receptors.\textsuperscript{50}

**Estrogen**

In premenopausal women, the ovaries are the main source of estrogen with the adrenal glands, adipose, hepatic and skin tissues also synthesizing small amounts. During gestation, the corpus luteum and placenta become significant contributors to estrogen
levels. The most common estrogens are estradiol, estriol, estrone, and estetrol. Estradiol is the prevailing form during gestation, with a shift to estriol becoming the dominate estrogen at term. Similar to progesterone, estrogen receptors (ERs) are intracellular ligand-activated transcription factors and exist as two major subtypes, ERα and ERβ. Human ERs are derived from separate genes, have different ligand binding affinities, and tissue distributions. Estrogen receptor complexes bind to estrogen response elements (EREs) in genes and promote a contractile phenotype by encoding for and increasing cellular sensitivity to CAPs and inflammatory cytokines. Estrogen’s additional genomic mechanisms include stimulating the production of PGs by maternal and fetal tissues, upregulating expression of OTR, and increasing gap junction formation to spread phasic contractions.

For most of pregnancy the human myometrium is refractory to estrogens, even though circulating estrogen levels increase at around mid-gestation and continue to rise gradually until birth. PR-B decreases uterine estrogen responsiveness by decreasing ERα expression. ERα expression is high in non-pregnant tissue and low in non-laboring term myometrium, but increases with the onset of labor, suggesting that functional estrogen activation is mediated by increased ERα expression. ERα expression positively correlates with the PR-A:PR-B expression ratio further supporting the theory that functional progesterone withdrawal induces functional estrogen activation. ERβ mRNA is low in non-laboring and laboring myometrium and this isoform’s role in parturition is being investigated further. Membrane G protein-coupled (GPCR) estrogen receptors have been identified in myometrial tissue of rodents and humans with activation
increasing depolarization, intracellular calcium levels, and myometrial contractile responses to oxytocin. \textsuperscript{62,63}

**Myometrial contraction physiology**

The myometrium is a myogenic, phasically active smooth muscle that is minimally active in the non-pregnant state and during most of gestation.\textsuperscript{36} Small regions of the uterus develop low-amplitude contractions that are not sufficient to cause cervical changes or initiate labor and these contractions slowly increase in frequency and strength as pregnancy progresses. During parturition, uterine contractions become synchronous with short durations and high amplitudes and involve the entire uterus.\textsuperscript{64} Active labor contractions build until the fetus is delivered, but exact feedback mechanisms regulating uterine contraction and relaxation are poorly understood. Myometrial electrical activity is closely coupled to contractile activity so that the amplitude, frequency, and duration of contractions correlate with the number of cells that are simultaneously active, their electrical burst frequency, and the duration of the bursts.\textsuperscript{36,64}

**Myometrial excitation**

The myometrium has the intrinsic ability to generate spontaneous electrical activity without the requirement of extrinsic neural or hormonal stimuli, though these factors can modulate them. Specialized pacemaker cells have been theorized as the initiators of activity in the myometrium, but these cells have not been identified. Different cells and channels have been evaluated on their ability to generate an action potential.\textsuperscript{36} Spontaneous activity may arise from many myometrial muscle cells as
initiation sites of electrical activity throughout the myometrium. For the uterus to contract as an entire organ, rather separate multicellular regions, coordination of individual cell activity is required. Depolarization of one cell is communicated to neighbors in a distinct pattern, so the wave of depolarization proceeds in a direction to allow for delivery of the fetus.\textsuperscript{64}

Over the course of gestation, the myometrial tissue transitions from a state of low intrinsic excitability, to one of high excitability that is vulnerable to stimulation.\textsuperscript{65} The resting membrane potential of myometrial tissue varies depending upon gestational state. Nonpregnant human myometrial membrane potential ranges from -40 to -60 mV, with potentials becoming more negative during pregnancy (-60 mV), and returning to approximately -45 mV at time of parturition.\textsuperscript{8,66–68} This change in resting membrane potential allows for easier generation of spontaneous actions potentials and their propagation through the myometrium in late pregnancy.

In the myometrium slow waves or gradual membrane depolarizations trigger action potentials that lead to spontaneous contractions.\textsuperscript{36} Spike action potentials are the predominate form in non-pregnant and early pregnancy, with Ca\textsuperscript{2+} the depolarizing and K\textsuperscript{+} the repolarizing currents.\textsuperscript{36} During gestation action potentials become complex with a plateau shape likely due to increased and prolonged Ca\textsuperscript{2+} influx and K\textsuperscript{+} channel expression changes. Excitation is coupled between cells through gap junctions in the plasma membrane composed mainly of Cx43. Gap junction expression and permeability increases late in pregnancy which allows for faster propagation of action potentials.\textsuperscript{36}

The myometrium expresses a plethora of different ion channels, and some have been determined to be essential for excitability and contraction, while the roles of others
are yet to be understood. An important contributor to cell excitability is the action of a leak current. Leak currents are constitutively active and allow a flow of cations across a cell membrane to depolarize the membrane and initiate action potential upstroke. The myometrial leak current is Na⁺-dependent, tetrodotoxin-insensitive, and Gd^{3+}-sensitive and depolarizes the potential to the extent for activation of L-type voltage gated calcium channels. The Na⁺-activated leak channel, non-selective (NALCN) channel allows passage of Na⁺, Ca^{2+}, K⁺, and Cs⁺ with decreased expression during mid-gestation and increased expression during labor in mice. NALCN provides ~50% of the myometrial leak current, as NALCN knockdown diminishes but does not complete abolish the leak current in human MSMCs. Knockout of NALCN in mouse models reveals reduced uterine excitability and increased incidence of abnormal labor, supporting its necessity for successful delivery.

Depolarization is caused by a rapid influx of calcium through these L-type calcium channels. The membrane potential gradually repolarizes by efflux of potassium ions, which reduces excitability and the duration of the action potential. The frequency of action potential discharge, the duration of the action potential train, and total number of cells activated determine the frequency, duration, and amplitude of uterine contractions. Gap junctions are sites of propagation or conduction of action potentials between cells, and are composed of a few to thousands of gap junction channels. Each gap junction channel is constructed of connexin proteins, that creates a pore and links up with another gap junction channel in an adjacent myocyte. This linkage of gap junctions couple myocytes together and create an electrical and metabolic syncytium. Gap junctions in the myometrium are composed primarily of Cx43. Coupling is poor
during most of pregnancy, as gap junction numbers are low or absent, but during labor
gap junctions begin to occupy a significant percentage of myocyte plasma membrane.\textsuperscript{73–75} Junctions begin to form approximately one day prior to the onset of labor and increase in
number and size during delivery. Conditional ablation of connexin 43 in mouse MSMCs
resulted in delayed parturition, demonstrating the importance of this protein in cell-to-cell
coupling for action potential propagation and uterine contractions.\textsuperscript{42}

**Myometrial force generation**

Consistent with other smooth muscle, human uterine smooth muscle relies on a
rise and fall of intracellular calcium concentration for contraction and relaxation. The rise
of intracellular calcium for myometrial contractions generally comes from two sources:
extacellular calcium influx through VGCC, and release of intracellular calcium from SR
stores.\textsuperscript{28–31,71,76} VGCCs, specifically the L-type calcium channels, provide the main route
for calcium entry into the myometrium\textsuperscript{8,9}, and create a steady-state calcium entry during
sustained depolarization.\textsuperscript{72} Internal store channels, such as ryanodine (RyR) and inositol
1,4,5-triphosphate (IP3R) receptor channels, amplify intracellular calcium levels through
release of calcium from internal stores.

Contraction and relaxation of the myometrium is regulated by phosphorylation
and dephosphorylation of the regulatory chain of myosin, which in turn is regulated by
intracellular calcium concentrations. Phosphorylation of myosin is affected primarily by
an increase in intracellular calcium concentrations. Increased cytoplasmic free calcium
results in the binding of calcium to the calcium binding protein, calmodulin (CaM).\textsuperscript{66,77}

After CaM has bound four calcium ions, it can bind to the CaM-binding domain
(CaMBD) on myosin light chain kinase (MLCK) resulting in activation of the enzyme.\textsuperscript{78}
MLCK specifically phosphorylates serine 19 on the regulatory myosin light chain, resulting in activation of actomyosin MgATPase. Actin–myosin cross bridging commences with movement of actin along myosin causing shortening of the muscle fibers and the development of force.

Calcium removal after contraction is necessary to induce relaxation and replenish the SR. This is achieved by a variety of mechanisms, including the closure of plasma membrane calcium channels, removal of calcium from the cytosol via plasma membrane Ca-ATPase (PMCA) and the sodium calcium exchanger (NCX), and movement of calcium back into the SR via the sarcoplasmic reticulum calcium ATPase (SERCA). A reduction in myosin light chain kinase activity can occur due to decreased intracellular calcium levels or via stimulation of the adenyl cyclase (AC)-cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) pathway. Myosin light chain phosphatase (MLCP) dephosphorylates myosin, allowing for detachment of actin from myosin cross bridging, and smooth muscle cell relaxation (Figure 2). In the myometrium generation of global calcium transients is accompanied by contractions. Rather than holding tone like vascular smooth muscle, when intracellular calcium concentrations decrease, relaxation occurs in the myometrium. Also unlike vascular smooth muscle, there is little evidence of force increasing without a change in intracellular calcium.

Potassium channels

Potassium efflux is responsible for maintaining the resting membrane potential and
repolarizing membrane action potentials.\textsuperscript{11,82,83} Potassium efflux produces relaxation through membrane hyperpolarization and impediment of calcium influx through VGCCs. Early studies with potassium channel blockers demonstrated an increase in contraction amplitude and duration due to prolongation of action potentials.\textsuperscript{84} Several types of potassium channels have been identified in the myometrium with the most well studied including the large-conductance calcium-activated potassium (BKCa) channels, ATP-sensitive potassium (K\textsubscript{ATP}) channels, voltage-gated potassium (K\textsubscript{V}) channels, and small-conductance calcium-activated potassium (SKCa, KCa2) channels.\textsuperscript{82,83,85}

BKCa channels are the predominant potassium channel identified in myometrial tissue.\textsuperscript{83} Their activity increases with membrane depolarization and/or increases in intracellular calcium concentrations\textsuperscript{83,86}, with activation primarily induced by voltage and not calcium. Down regulation of BKCa channel transcripts occurs during pregnancy.\textsuperscript{87}

K\textsubscript{ATP} channels are ATP-sensitive inward rectifying potassium channels that regulate cellular responses by coupling cell metabolism with membrane potential.\textsuperscript{88} The potassium channel family Kir6 makes up the pore-forming component of the channel, with ATP sensitivity, pharmacological properties, and trafficking regulated by a sulfonylurea receptor.\textsuperscript{82,85} K\textsubscript{ATP} channels are expressed at higher levels in non-pregnant myometrium with a two to three fold downregulation in late gestation.\textsuperscript{83,88} K\textsubscript{V} channels are presumed to be responsible for repolarizing membrane action potentials and regulating membrane potential.\textsuperscript{89} The K\textsubscript{V}4 subfamily has shown to play the most predominant role in pregnant myometrium and generates a rapidly inactivating potassium current in rat pregnant myometrium.\textsuperscript{82} Within the subfamily K\textsubscript{V}4.2 channel
expression increased before labor, whereas $K_v$4.1 and $K_v$4.3 expression declined during gestation, suggesting a possible hormonal regulation of channel expression.\textsuperscript{82}

\textbf{Figure 2. Diagram of uterine smooth muscle contractile mechanisms.} A leaky $Na^+$ current (demonstrated by NSCC) depolarizes the membrane potential enough to meet the threshold potential and open voltage-gated, L-type calcium channels (VGCC). This causes rapid $Ca^{2+}$ influx, which binds to CaM and activates MLCK, leading to phosphorylation of the myosin regulatory light chains, enabling interaction of myosin with actin, cross-bridge cycling and force development. Membrane potential depolarization activates $K_v$ channels and $Ca^{2+}$ activates $KCa$ channels, both causing $K^+$ efflux and membrane repolarization, closing VGCC. Cytosolic $Ca^{2+}$ levels continue to decrease due to closure of membrane $Ca^{2+}$ channels, removal of $Ca^{2+}$ from the cytosol via the PMCA and NCX, and movement of $Ca^{2+}$ back into the SR via the SERCA. MLCP dephosphorylates myosin, allowing for detachment of actin from myosin cross bridging, and smooth muscle cell relaxation, until the next action potential. NSCC, nonselective cation channel; VGCC, voltage gated $Ca^{2+}$ channel; CaM, calmodulin; MLCK, myosin light chain kinase; $K_v$, voltage gated $K^+$ channel; $KCa$, $Ca^{2+}$-activated $K^+$ channel; PMCA, plasma membrane $Ca^{2+}$-ATPase; NCX; $Na^+-$-$Ca^{2+}$ exchanger; SR, sarcoplasmic reticulum; SERCA; sarcoplasmic reticulum calcium ATPase; MLCP; myosin light chain phosphatase.
Small-conductance calcium-activated potassium channels

Bond et al. identified a relatively unstudied potassium channel as a major player in the regulation of uterine contractility. KCa2.3, or SK3, is one of three subtypes of small conductance calcium-activated potassium channels. A regulatory cassette with a site-specific tetracycline/doxycycline-inhibitable transactivator protein that promoted gene expression of KCa2.3 channel subunits, was inserted into mice. This doxycycline (DOX)-sensitive genetic switch also allowed for titration of KCa2.3 expression levels through administration of dietary doxycycline, which would suppress KCa2.3 expression. Female mice that overexpressed KCa2.3 channels (SK3^{T/T}) demonstrated normal mating behavior, fertility, and carried pups to term. However, in seven of the ten litters, delivery was protracted and some of the mothers and pups did not survive. Autopsy of mothers that died during parturition revealed pups blocking the birthing canal. Eleven pregnant mice that overexpressed KCa2.3 were given dietary doxycycline (SK3^{DOX}) to normalize KCa2.3 levels. In these mice delivery occurred in a normal fashion without injuries to dams or pups. This study revealed KCa2.3 overexpression compromised labor and identified KCa2.3 channels as an imperative regulator of myometrial contractility.

SKCa channels are present in non-excitable and excitable tissues, and their high calcium sensitivity (0.3-0.5 µM), lends to its role in the regulation of signaling pathways involving calcium. Three different isoforms of SKCa channels (KCa2.1-2.3, SK1-3) are produced by three different genes (KCNN1-3), with channel isoforms sharing a high overall structural homology. SKCa channels are six transmembrane channels composed of four alpha subunits (Figure 3). The potassium selective filter and the pore forming unit are located between the fifth and sixth transmembrane domain.
SKCa channels is voltage-independent and relies exclusively on calcium binding to constitutively associated CaM\textsuperscript{13–17,92}, located at the CaMBD on the C-terminus of each alpha subunit.\textsuperscript{13,14,24,90,92,93} The C-lobe of CaM mediates the constitutive interaction with the CaMBD of SKCa channels.\textsuperscript{94} Calcium activation of SKCa channels is the result of calcium binding to the two EF hands in the N-lobe of CaM.\textsuperscript{13,16,17,94} When calcium binds to CaM, it undergoes a conformation change\textsuperscript{17,24,90}, exposing residues that interact with the CaMBD on an adjacent channel subunit.\textsuperscript{16,31,90} This shifts the channel from a monomeric structure into a stable dimer of dimers complex.\textsuperscript{14–16} The channels calcium sensitivity is regulated through the association of a kinase/phosphatase pair.\textsuperscript{14,17,24,90,93} Casein kinase 2 (CK2) and protein phosphatase 2A (PP2A) mediate the phosphorylation and dephosphorylation of CaM’s threonine 80 (T80)\textsuperscript{14,17,24,90,92}, with the phosphorylated state being less calcium sensitive.\textsuperscript{14,90,92,93} The regulatory actions of CK2 and PP2A are state-dependent; CK2 only phosphorylates T80 when the channel is closed, and PP2A only dephosphorylates when the channel is open.\textsuperscript{14,92} Channel deactivation is the reverse process with calcium dissociating from CaM, and the channel shifting back into a closed state.\textsuperscript{13,90,95} SKCa channel activity largely follows that of free calcium near the channel, thus the kinetics of the macroscopic current depend on the source and location of calcium.\textsuperscript{14}

KCa2.3 are expressed across a range of tissues, with high abundance in regions of the brain, but also present at significant levels in peripheral tissues rich in smooth muscle.\textsuperscript{96} KCa2.3 channels are present in mouse\textsuperscript{12,21,27}, rat\textsuperscript{18}, and human\textsuperscript{20,23,26,97}.
Figure 3. Diagram of KCa2.3 channel with associated calmodulin and regulatory proteins, CK2 and PP2A. KCa2.3 channels are six transmembrane channels composed of four alpha subunits and activated by intracellular calcium binding to a constitutively associated calmodulin. The channel’s calcium sensitivity is regulated through the phosphorylation and dephosphorylation of a calmodulin threonine subunit by CK2 and PP2A (ref).
myometrium. KCa2.3 expression patterns in rat\textsuperscript{18} and human\textsuperscript{20} uterine tissue demonstrated the greatest abundance in the smooth muscle layers of the myometrium, and less abundant in the glandular epithelium and stroma of the endometrium. KCa2.3 channel transcript and protein expression levels are not consistent between mammals. Mouse uterine tissue reveals a decrease in channel transcript\textsuperscript{22} and channel protein expression\textsuperscript{22,27} during pregnancy beginning at mid-gestation. Rat uterine tissue showed consistent KCa2.3 channel transcript and protein expression throughout gestation, without the decrease seen in mouse tissue.\textsuperscript{18} KCa2.3 channel transcript and expression\textsuperscript{20,23,87,97} are both decreased in late pregnant human uterine tissue compared to non-pregnant tissue.

Further studies with KCa2.3 overexpression assessed the channels contribution to murine uterine contractility. Brown \textit{et al.}\textsuperscript{21} and Pierce \textit{et al.}\textsuperscript{22} confirmed KCa2.3 expression was substantially increased in SK3\textsuperscript{T/T} mice. Immunofluorescence revealed distinct KCa2.3-positive staining in longitudinal bundles of mouse myometrium, with SK3\textsuperscript{T/T} mice having greater channel signal compared to SK3\textsuperscript{DOX} mice in the myometrial samples.\textsuperscript{21} SK3\textsuperscript{T/T} uterine tissue strips had reduced force generation in myographical studies when compared to wild type (WT) and SK3\textsuperscript{DOX} mice, indicating that KCa2.3 overexpression diminishes uterine contractility.\textsuperscript{21,22} Induction of premature labor led to delivery within 24 hours for WT and SK3\textsuperscript{DOX} mice with SK3\textsuperscript{T/T} mice failing to complete delivery.\textsuperscript{21} These findings support the concept that KCa2.3 assists in negative feedback of uterine contractility and is crucial to effective parturition.
Modulation of SKCa channels

Fine tuning of SKCa relaxant effects is possible though pharmacological gating modulation of the channel. Gating modulation of SKCa channels can occur through three mechanisms: interaction with the pore (e.g., pore blockers such as apamin), modification of the cytosolic calcium sensitivity of the channel (e.g., positive and negative modulators), and amphiphilic structures acting by integration into the membrane.\textsuperscript{11}

Apamin is a toxin isolated from honeybee venom that is a highly selective SKCa channel blocker. Apamin is not a classic pore blocker, in that it does not physically occlude the pore, rather it works through allosteric mechanisms to collapse the outer pore of the channel.\textsuperscript{14,24,93} Apamin’s ability to block SKCa channels is different between channel subtypes, with KCa2.2 displaying the highest sensitivity, KCa2.3 a moderate sensitivity, and KCa2.1 the least.

Two main classes of chemotypes make up positive allosteric modulators (PAMs) of SKCa channels. The bicyclic heterocyclic riluzole family activates all SKCa and IKCa channels, with some also activating ion channels of other classes.\textsuperscript{93} The cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]amine (CyPPA) family is highly selective as they activate KCa2.2 and KCa2.3 channels and are completely inactive on KCa2.1 and KCa3.1.\textsuperscript{25,93} While these families are structurally distinct, a PAM binding pocket was identified between calmodulin and CaMBD on the SKCa interface, with the ability to accommodate structurally diverse small molecules.\textsuperscript{15,24,25,93} Binding of a small-molecule to the PAM binding pocket causes reorganization of side chains on the SKCa channel and calmodulin.\textsuperscript{15,24} Side channel rearrangement is theorized to change calmodulins interaction with channel regulatory proteins and alters channel state. This
action shifts the calcium activation curve toward lower concentration of calcium in a concentration-dependent manner.\textsuperscript{13,16,17,24,25,90,92,93,98}

Early myometrial studies with CyPPA employed myographical measurements of contraction force and frequency of murine isolated myometrial strips. Myometrial samples were taken from mice in varying states of gestation, specifically non-pregnant (NP), day 10 (D10) and day 16 (D16) of pregnancy. These studies found increasing concentrations of CyPPA had an ambiguous impact on uterine contraction frequencies but significantly decreased contraction amplitudes. Contractions suppressed by CyPPA could be restored with the addition of apamin.\textsuperscript{27} A preterm labor model was employed to assess the effect of CyPPA in these conditions, and included contraction induction via RU486 in pregnant mice. After phasic contractions developed a peritoneal injection of CyPPA was administered to treatment group dams. Mice treated with CyPPA had a 3.4-hour delay of preterm birth with increased pup retention when compared to the control group.\textsuperscript{27} A small study utilizing non-pregnant and pregnant human myometrial samples found CyPPA caused a concentration-dependent reduction of uterine contractility in both gestational groups with concentrations of 60 μM abolishing contractions in both groups.\textsuperscript{26}

**Internal calcium store channels**

The major source of calcium for contractions arises from L-type calcium channels, but intracellular calcium release channels play a part by amplifying free cytosolic calcium concentration through calcium-induced calcium release (CICR) from internal stores. Movement of cytosolic calcium into the SR after activation of the SERCA pump leads to internal store replenishment. IP3Rs are the predominate intracellular
calcium release channel in the myometrium and control release of SR calcium after
activation.\textsuperscript{75,99–102} IP3R channels (IP3R1, IP3R2, IP3R3) are assembled from four large
subunits, each with a single IP3 binding site.\textsuperscript{31,100–103} IP3Rs are CICR receptors
commonly found in clusters in the SR membrane. Binding of an IP3 molecule to each
subunit of the channel exposes a calcium binding site. Free calcium from the cytoplasm
binds to each open calcium binding site allowing opening of the channel and calcium
release from stores (Figure 4).

Activation of an IP3R begins when intracellular IP3 binds to a pocket located
between the a and b domains of the IP3-binding core (IBC).\textsuperscript{100} Priming of the channel is
complete after all four subunits have bound IP3 (Figure 4), exposing the calcium binding
sites.\textsuperscript{90,91,94} Once calcium binds, a conformational change is triggered and the channel
opens.\textsuperscript{91,94} Strong and persistent extracellular signals can activate multiple IP3Rs to create
calcium waves.\textsuperscript{66,91,93} Calcium waves are further propagated as higher concentrations of
IP3 prime more IP3Rs to respond to calcium in their local environment and increase
channel opening probability. IP3Rs ability to activate neighboring IP3Rs, through
calcium release, demonstrates the importance of subcellular location in shaping
intracellular calcium signaling.\textsuperscript{100,102,103} IP3R calcium gating is biphasic, with low
calcium concentrations acting in a stimulatory manner, and higher concentrations
inhibiting channel opening.\textsuperscript{100} A decrease in IP3 generation triggers the IP3Rs to close,
diminishing intracellular calcium concentrations and allowing internal calcium stores to
refill.\textsuperscript{100}

IP3 generation relies on ligand-operated G\textsubscript{q}-protein coupled receptors (G\textsubscript{q}PCR)
activating phospholipase C-\textbeta (PLC\textbeta) to cleave the membrane phospholipid
Figure 4. Calcium-induced calcium release from IP3R channels. IP3Rs are the predominate intracellular calcium release channel in the myometrium and amplify cytosolic Ca\(^{2+}\) levels through CICR from internal stores. IP3R channels are assembled from four large subunits, each with a single IP3 binding site. When an IP3 molecule binds to each subunit, Ca\(^{2+}\) binding sites are exposed. Ca\(^{2+}\) binding triggers a conformational change, and the channel opens, releasing Ca\(^{2+}\) from internal stores into the cytosol. IP3R, inositol 1,4,5-triphosphate receptors; CICR, calcium induced calcium release.
phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and IP3 (Figure 5).\textsuperscript{66,67,104,105} DAG activates protein kinase type C (PKC) which effects MLCP activity through phosphorylation\textsuperscript{106} and IP3 controls mobilization of calcium from SR stores.

**Agents that increase IP3 generation**

**Oxytocin**

Oxytocin is a peptide hormone that is synthesized in the hypothalamus and stored in the axon terminals of the posterior pituitary. During gestation the uterus, placenta, and decidua also synthesize oxytocin. Oxytocin is released discontinuously from the posterior pituitary and circulates as a free peptide in the bloodstream. Oxytocin is typically controlled by neural mechanisms but hormones, such as estrogen, can stimulate oxytocin release as well. Oxytocin receptors (OTR) are present in human myometrium, endometrium, and decidua with expression of OTR increasing during late pregnancy and early labor, allowing increased sensitivity of myometrial tissues to slight elevations of oxytocin.\textsuperscript{66,107,108} The expression of myometrial OTR is high and uniform in the fundus and corpus, and sharply declines in the lower uterine segment, isthmus, and cervix.\textsuperscript{109}

The OTR is part of the Class I G-protein coupled receptor family and responds to oxytocin binding in the myometrium by increasing cytosolic calcium levels through multiple mechanisms. These include calcium entry via L-type calcium channels\textsuperscript{110,111}, increased IP3 generation through stimulation of the PLC pathway\textsuperscript{112-116} (Figure 5), and limiting calcium extrusion by inhibiting Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange and Ca\textsuperscript{2+}-ATPase.\textsuperscript{117,118} OT activates RhoA which regulates acto-myosin coupling and calcium sensitivity of smooth
Figure 5. GPCR mechanism of IP3 generation. Ligand-bound GPCR activates PLCβ which cleaves PIP2 into DAG and IP3.
muscle contractile proteins.\textsuperscript{106} Activated RhoA further activates Rho kinase which phosphorylates the myosin-binding subunit of MLCP leading to inhibition of myosin light chain dephosphorylation.\textsuperscript{119–121} OT activation also stimulates uterine contractility by increasing release of PGF2a and arachidonic acid.\textsuperscript{114,115} Continuous oxytocin stimulation can lead to loss of oxytocin receptors, demonstrated decreased number of oxytocin binding sites and the very low receptor mRNA concentrations, culminating in receptor desensitization.\textsuperscript{122–124}

**Prostaglandins**

Prostaglandins (PGs) are lipids derived from arachidonic acid and synthesized within human fetal amnion and chorion membranes and maternal decidua. PGs exert their effect via G-protein coupled prostanoid receptors, of which many exist. PG receptors include those for PGF2a (FP), PGE\textsubscript{2} (EP\textsubscript{1-4}), thromboxane (TxA\textsubscript{2}), prostacyclin (IP), and PGD (DP). Each PG receptor initiates a different intracellular signaling pathway, with some PGs promoting a contractile response (FP, EP\textsubscript{1}, and EP\textsubscript{3}) and others a relaxing effect (EP\textsubscript{2}, EP\textsubscript{4}, IP, and DP).\textsuperscript{104} The two major stimulating PGs present in human myometrium are PGF2a and PGE, with levels of these PGs increasing as gestation advances.\textsuperscript{104} PGF2a increases intracellular calcium levels by stimulating IP3-mediated SR calcium release\textsuperscript{114–116} and increasing calcium influx through activation of NSCCs. PGs also stimulate myometrial gap junction formation, allowing for sharing of intracellular resources into neighboring MSMCs.\textsuperscript{125}
KCa channels and internal calcium store channels

Studies over the past decade have shown activation calcium for KCa channels comes from a preferential source and can arise from plasma membrane calcium channels or internal store release channels. Close proximity or local coupling has been found to enhance plasma membrane channel activation by SR calcium release through local IP3Rs. In rat and mouse cerebral artery smooth muscle cells, IP3-mediated calcium release activates BKCa by elevating BKCa channel calcium sensitivity through local molecular coupling.126 Erythrocyte lysate releases calcium from IP3-sensitive intracellular stores and activates BKCa channels in rat basilar arterial myocytes via increased cytoplasmic calcium levels.127 Ryanodine receptor channels also release calcium from internal stores in smooth muscle SR and control the diameter of small myogenic arteries through activation of BKCa channels.128

Calcium influx via vanilloid transient receptor potential subtype-4 (TRPV4) channels selectively activate KCa3.1 and KCa2.3 channels in mouse mesenteric arteries33,35, pulmonary microvascular endothelial cells (PMVECs), and pulmonary arterial endothelial cells (PAECs).32 Tonic activity of endothelial KCa2.3 channels creates a sustained hyperpolarization of the endothelial membrane potential, which is then communicated to adjacent smooth muscle.129 This forms a positive feedback loop that regulates membrane potential through increased intracellular calcium, either through calcium entry or release from internal stores, activation of KCa channels and endothelium-derived hyperpolarization.32,35

Coupling of TRPC3 channels to IP3R in arterial myocytes leads to T-type calcium channel activation and vasoconstriction. Close spatial proximity between IP3R1 and
TRPC3 establishes this isoform-selective functional interaction.\textsuperscript{130,131} Additionally IP3 was found to constrict cerebral arteries via an IP3R-mediated TRPC3 activation that was independent of SR calcium release.\textsuperscript{132} Further investigation into this relationship found TRP3 binds calcium-CaM at a site that overlaps with the IP3R binding domain.\textsuperscript{133} IP3R-mediated calcium release also activates TRPC1 channels in aortic myocytes\textsuperscript{134} and portal vein smooth muscle cells.\textsuperscript{135} In cerebral artery smooth muscle cells calcium release for SR stores is required for TRPM4 activity.\textsuperscript{136}

A variety of neuronal cells have revealed a slow spontaneous current through SKCa channels that are activated by RyR mediated calcium release from internal stores.\textsuperscript{14} Electron microscopy of Purkinje cell dendrites found colocalization of RyR and SK2 channels. Coupling of RyR and SK2 channels with VGCC channels is theorized to underlie generation of afterhyperpolarization in these cells. Calcium influx through Cav channels is required to induce CICR of internal stores through RyR, which then activates SKCa channels.\textsuperscript{14} Spine SK channels are activated by calcium influx through R- and L-type calcium channels as well as calcium released from internal stores through IP3R.\textsuperscript{14}

The basis of this functional relationship includes calcium influx through a plasma membrane calcium channel, that increases cytosolic calcium and opens internal store calcium channels, either IP3 or RyR. Calcium release from internal stores is directed at KCa channels due to their proximity, in these ER/SR projections. These studies demonstrate the potential for local coupling of SKCa channels with internal store channels and plasma calcium channels for regulation of calcium events. These studies support the concept of internal stores and membrane KCa channel coupling as a primary mechanism of calcium feedback control.
Myometrial specific evidence for this concept came from unpublished rodent studies by our laboratory, assessing CyPPAs ability to reduce oxytocin stimulated contractility (Figure 6). Non-pregnant murine myometrial strips were pretreated with CyPPA, to increase calcium sensitivity of KCa2.3, with increasing amounts of oxytocin then applied. In the vehicle treated group, every addition of oxytocin increased uterine contractility, but in the CyPPA pretreated group, oxytocin failed to increase mouse myometrial contractility. These data support a role for internal calcium stores on KCa2.3 activation and myometrial negative feedback.

**Preterm labor**

One in ten babies is born prematurely in the United States and despite advancing knowledge of risk factors and introduction of medical interventions, this incidence remains consistent. Preterm birth (PTB) is defined as delivery prior to 37 weeks gestation and is the leading cause of neonatal death and the second cause of childhood death before the age of five. Infants who are born preterm are at a higher risk for lifelong pulmonary, cardiac and neurodevelopmental disorders, as well as short-term complications due to immaturity of organ systems. The United States has the greatest incidence of PTB amongst high resource countries and is the only high-income country in the top ten countries for number of preterm births in 2014.

Spontaneous PTB accounts for approximately 70% of preterm deliveries, with the remaining 30% accounting for medically induced birth due to maternal or fetal complications. Spontaneous PTB may be idiopathic, or triggered by preterm premature rupture of membranes (PPROM), or inflammation of the gestational tissues.
Figure 6. CyPPA suppression of spontaneous and oxytocin-induced contractions of isolated mouse uterine strips. Isometric myography recordings of mouse uterine tissue treated with either 10 μM CyPPA (A) or DMSO vehicle (B). Following treatment with CyPPA, oxytocin failed to amplify uterine contractions and further dampened contractions at 1 μM. CyPPA-suppressed contractions are restored by addition of the KCa2.3 inhibitor apamin (0.6 μM).
Additional medical risk factors for PTB include hypertension, placental abruption, placenta previa\textsuperscript{140}, preeclampsia\textsuperscript{141}, eclampsia, previous preterm birth, previous delivery of a low birth weight infant, and low pregnancy weight gain.\textsuperscript{142,143} Non-medical risk factors of preterm birth include ethnicity\textsuperscript{144}, maternal circadian dysregulation\textsuperscript{145–147}, and air pollution\textsuperscript{148,149}.

Identifying women who present with preterm contractions and will eventually deliver preterm is a challenge for providers. The diagnosis of preterm labor generally is based on presentation of regular uterine contractions accompanied by a change in cervical dilation, effacement, or both, or initial presentation with regular contractions and cervical dilation of at least 2 cm.\textsuperscript{150}

**Management of preterm labor**

Therapies associated with improved neonatal outcomes include antenatal corticosteroids for maturation of fetal lungs and the targeted use of magnesium sulfate for fetal neuroprotection.\textsuperscript{150} The American College of Obstetrics and Gynecologists (ACOG) recommends a single course of corticosteroids for pregnant women between 24 weeks and 34 weeks of gestation who are at risk of delivery within 7 days.\textsuperscript{150} Preferred corticosteroids include intramuscular administration of either two 12-mg doses of betamethasone given 24 hours apart or four 6-mg doses of dexamethasone given every 12 hours.\textsuperscript{150} Magnesium sulfate has shown to reduce the risk and severity of cerebral palsy in surviving infants if administered when birth is expected before 32 weeks gestation.\textsuperscript{151}

ACOG supports short-term use of tocolytic medications in women with preterm labor to suppress delivery long enough for the administration of antenatal steroids and
magnesium sulfate, as well as transportation to a tertiary facility if necessary. Tocolytic medications work by decreasing uterine tissue excitability and contractility to cause uterine relaxation.

**Tocolytic agents.**

The importance of certain ion channels and receptor stimulated pathways to uterine contractility is not a new concept. Tocolytic medications either inhibit ion channels or block a stimulus to a second messenger pathway which can lead to off-target effects and potential maternal and fetal adverse reactions. Tocolytic medications have not been shown to reduce the rate of preterm delivery or improve neonatal outcomes. Tocolytic agents are given to suppress preterm labor long enough, for administration of antenatal steroids, to accelerate fetal lung development. Only 80% of preterm labor can be suppressed with tocolytics and at most for five days, which is merely a stop gap and not a solution to the problem. The physiologic mechanisms underlying tocolytic medications vary from systemic to targeted approaches, but their effectiveness is limited and a new tocolytic medication has not been introduced in 35 years.

Beta mimetics, such as terbutaline and historically ritodrine, are beta-adrenergic receptor (β2AR) agonists that stimulate adenyl cyclase in myometrial cells through GsPCR activation. GsPCR increases cAMP production, decreases intracellular calcium and activates PKA. PKA inhibits PLCβ and VGCCs, decreasing intracellular calcium levels. PKA also phosphorylates MLCK, altering MLCKs affinity for calcium-calmodulin, reducing phosphorylation of myosin light chains and promoting myometrial relaxation.
Oxytocin receptor antagonists, such as atosiban, competitively bind oxytocin receptors in the plasma membrane of myometrial cells preventing oxytocin-induced calcium influx and IP3 generation.\textsuperscript{125}

Magnesium sulfate is a general smooth muscle relaxant. Magnesium sulfate acts as a calcium competitor by blocking entry of calcium via L- and T-type VGCC, which reduces action potential generation and intracellular calcium levels.\textsuperscript{68,125} Magnesium also enhances smooth muscle relaxation by enhancing KCa channel calcium sensitivity and decreasing SR calcium release via decrease of PLC\(\beta\) generation.\textsuperscript{68,104,159–161}

Prostaglandin inhibitors, such as ketorolac and indomethacin, block cyclooxygenase (COX) enzyme from converting arachidonic acid into prostaglandin H2, and eventually PGF\(2\alpha\) and PGE.\textsuperscript{162,163}

Calcium channel blockers, such as nifedipine, reduce the influx of calcium into myometrial cells by blocking VGCC.\textsuperscript{125,155,164} Calcium influx from the extracellular environment is largely responsible for the increase in intracellular calcium that is required to activate the actin-myosin contractile machinery. Inhibition of plasma membrane calcium channels can also prevent action potential propagation.\textsuperscript{104,155}

As current tocolytic methods are not effective and only suppress labor for a few days, the possibility of a tocolytic that enhances an innate uterine relaxation feedback system should be investigated. Rather than stopping a stimulus to contraction, fine tuning of a feedback system could be employed to relax the uterus.
CHAPTER III

MATERIALS AND METHODS

General materials and methods

Patient selection

Approval for the collection of uterine biopsies was obtained from the University of South Alabama’s Institutional Review Board. Fully informed written consent was sought from all patients participating in this study by either research personnel or medical providers from the Department of Obstetrics and Gynecology. Tissue was obtained from women undergoing a hysterectomy for a gynecological condition, or those having a cesarean section for delivery of a child.

Human tissue samples

Full thickness uterine samples from non-pregnant women (mean age 41.4; range 27 - 69 years; \( n = 22 \)) were collected in the operating room at the time of hysterectomy and excised from the upper aspect of the lower uterine segment. In the operating theater, samples were placed in HEPES buffer and immediately transported to the lab for processing. All studies performed with non-pregnant samples were conducted within 24 hours of excision time. Indications for hysterectomy included: dysmenorrhea, uterine
fibroids, endometriosis, uterine prolapse, and risk of cancer. Exclusion criteria for patient participation included: patient under the age of 18 years or presence of an infection outside the limitations of a BSL-2 laboratory. Table 1 describes the demographic and clinical data of included non-pregnant patients.

<table>
<thead>
<tr>
<th>Table 1. Demographic and clinical data of non-pregnant women.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, years</strong></td>
</tr>
</tbody>
</table>
| **Ethnicity** | Caucasian: 12  
| | African American: 9  
| | Latino/Hispanic: 1 |
| **Indications for hysterectomy**  
(subject may have multiple reasons) | Dysmenorrhea: 19  
| | Uterine fibroids: 6  
| | Endometriosis: 2  
| | Risk of cancer: 2  
| | Uterine prolapse: 2 |
| **Additional gynecological history**  
(subject may have multiple conditions) | History of abnormal PAP smear: 9  
| | Ovarian cysts: 5 |

Full thickness uterine samples from pregnant women (gestational age range 28+6 - 39+3 weeks; mean maternal age 29.1; range 21 - 45 years; n = 19) were collected in the operating room at the time of cesarean section and excised from the upper edge of the lower uterine segment incision (except for one patient with extensive abdominal adhesions that required the sample be taken from the lower edge), immediately after the baby was born. Biopsies included term, non-laboring (n = 14), preterm, non-laboring (n = 3), and preterm, laboring (n = 2), from patients undergoing a repeat cesarean section (n = 17) or a first-time cesarean section (n = 2). In the operating theater, samples were placed in HEPES buffer and stored in 4°C refrigerator until collected by research personnel. All studies performed with pregnant samples were conducted within 48 hours of excision time. Indications for cesarean section included: breech presentation, previous cesarean
section, maternal request, premature rupture of membranes, preeclampsia/eclampsia, gestational hypertension, and previous myomectomy. Exclusion criteria for patient participation included: patient under the age of 18 years or presence of an infection outside the limitations of a BSL-2 laboratory. Table 2 describes the demographic and clinical data of included pregnant patients.

<table>
<thead>
<tr>
<th>Maternal age, years</th>
<th>21 - 45 years (mean 29.1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethnicity</td>
<td></td>
</tr>
<tr>
<td>Caucasian: 10</td>
<td></td>
</tr>
<tr>
<td>African American: 8</td>
<td></td>
</tr>
<tr>
<td>Asian: 1</td>
<td></td>
</tr>
<tr>
<td>Gestational age at delivery, weeks</td>
<td>28+6 - 39+3 weeks (mean 37+1)</td>
</tr>
<tr>
<td>Laboring status</td>
<td></td>
</tr>
<tr>
<td>Term, non-laboring: 14</td>
<td></td>
</tr>
<tr>
<td>Preterm, non-laboring: 3</td>
<td></td>
</tr>
<tr>
<td>Preterm, laboring: 2</td>
<td></td>
</tr>
<tr>
<td>Indications for cesarean section (subject may have multiple indications)</td>
<td>Previous cesarean: 16</td>
</tr>
<tr>
<td>Maternal request: 1</td>
<td></td>
</tr>
<tr>
<td>Breech presentation: 1</td>
<td></td>
</tr>
<tr>
<td>Preeclampsia/eclampsia: 2</td>
<td></td>
</tr>
<tr>
<td>Premature rupture of membranes: 2</td>
<td></td>
</tr>
<tr>
<td>Gestational hypertension: 1</td>
<td></td>
</tr>
<tr>
<td>Previous myomectomy: 1</td>
<td></td>
</tr>
<tr>
<td>Additional gynecological history (subject may have multiple conditions)</td>
<td>History of abnormal PAP smear: 6</td>
</tr>
<tr>
<td>Uterine fibroids: 3</td>
<td></td>
</tr>
<tr>
<td>Endometriosis: 1</td>
<td></td>
</tr>
</tbody>
</table>

**Materials**

Common solutions used throughout these studies included HEPES and PBS buffer. Phosphate-buffered saline (PBS), pH 7.4 containing (in mM): 135 NaCl, 1.3 KCl, 3.2 Na₂HPO₄, and 0.5 KH₂PO₄. HEPES buffer, pH 7.4 containing (in mM): 134 NaCl, 6 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES acid, 10 glucose.
Figure 7. Dissection and allocation of human uterine tissue. (A) Tissue is excised from the anterior aspect of the uterus. (B) Tissue is transported to the lab and washed in HEPES buffer. (C) Portions of the myometrium without or without endometrium are dissected out for freezing and immunofluorescent staining. (D) Myometrium is sectioned further into strips and (E) sewn into rings with suture. (F) Uterine rings are mounted around myograph pins, with one pin attached to a force transducer for measurements.
**Immunofluorescent microscopy**

Samples of fresh human uterine tissue were rinsed with HEPES buffer, and sections of tissue including a combination of myometrium or myometrium and endometrium were dissected (Figure 7). Sections were placed in a Tissue-Tek cryomold (Sakura Finetek) and covered with Tissue-Tek O.C.T. compound (Sakura Finetek). Tissue was then flash frozen in the cryomolds by liquid nitrogen. Samples were moved to the -80°C freezer for storage. Frozen samples were sliced via cryostat into 10 micron or 20 micron thick sections and placed on slides by lab personnel of the University of South Alabama Pathology Department. Tissue slices were fixed with 5% paraformaldehyde and permeabilized with 0.2% Triton X-100 (Sigma) in PBS. Tissues were blocked with 2% BSA and 10% normal goat serum in 0.2% Triton X-100 (Sigma) in PBS, then incubated with KCa2.3 primary antibody (rabbit, anti-KCNN3; Alomone Labs; 1:250) overnight at 4°C. The following day tissue was stained with secondary antibody (goat anti-rabbit, Alexa Fluor 568; Molecular Probes, Invitrogen; 1:500). For dual staining images the tissue were then incubated with IP3R primary antibody (monoclonal, mouse, recombinant anti-IP3R; Abcam; 1:250) overnight at 4°C. The next day tissue was stained with secondary antibody (goat anti-mouse, Alexa Fluor 488; Molecular Probes, Invitrogen; 1:500). Tissues were stained for nuclear visualization (NucBlue; ThermoFisher) for ten minutes and then mounting media and coverslips were applied. The specificity of secondary antibodies were tested by staining myometrial samples with only the red (Alexa Fluor 568) or green (Alexa Fluor 488) secondary antibodies. Samples were viewed with a Nikon A1 confocal and secondary only controls demonstrated no fluorescent signal. Negative control images for red and green secondary antibodies can be
found in Figure 40 in Appendix A. Tissues were imaged with a Nikon A1 confocal microscopy with imaging conducted under identical conditions for all preparations.

**Tissue myography**

**Uterine ring preparations**

Fresh human uterine tissue was washed in HEPES buffer and small strips measuring 8-10 mm in length, 3 mm in width and 3 mm in depth were created from myometrial tissue. These strips were sutured with 6-0 Prolene polypropylene suture (Ethicon) in an end-to-end fashion to create myometrial rings. Myometrial rings were mounted around the myographical pins, with one pin connected to a force transducer and the other to an adjustable micrometer (Figure 7). These pins were suspended in an organ bath containing 5 mL of prewarmed (37ºC) and gassed (95% O₂, 5% CO₂). All experiments were conducted under these conditions.

**Myographical recordings**

Two myography machines were used: a Danish Myo Technology (DMT) multi myograph system model 610M and a DMT multi myograph system model 620M. Once myometrial rings were mounted, they were incrementally stretched until they held 1-4 mN of tone. The rings were allowed to rest until they developed spontaneous contractions. Following equilibration, uterine rings exhibited a regular pattern of spontaneous contractions and therefore it was not necessary to induce contractions with a chemical stimulant. Contraction-response experiments were initiated after four
spontaneous and consistent contractions occurred. Uterine rings that did not develop spontaneous contractions were excluded from experiments.

**Drugs**

Concentration-response curves for oxytocin, prostaglandin F2a, m-3M3FBS, and U73122 were conducted on non-pregnant and pregnant tissue to confirm dosages used in other studies and determine concentrations for drugs not previously used on myometrial tissue. Concentration-response analysis is located in Figure 41 in Appendix A. Other drugs including CyPPA, apamin, cyclopiazonic acid, and nifedipine were used in concentrations referenced from previous internal and external studies.

CyPPA, prostaglandin F2a, m-3M3FBS, and U73122 (each dissolved in DMSO) were purchased from Tocris. Oxytocin and cyclopiazonic acid (each dissolved in DMSO) were purchased from Abcam. Apamin (dissolved in DMSO) was purchased from R&D systems. Nifedipine (dissolved in DMSO) was purchased from Sigma. Exogenous activators and inhibitors were added separately to the tissue bath in 20-minute intervals.

**Statistical analysis**

Analogue signal output was digitalized with a PowerLab and visualized and analyzed using LabChart software (both AD Instruments). Independent contractions are described as any deflection of >10% of maximum contraction amplitude (for the individual recording) and then drops below 20% of maximum peak. If a waveform has two peaks and drops to below 20% of the max amplitude, it will be counted as two contractions. Parameters assessed included frequency (contractions/minute), amplitude (gram tension; mN), and area under the curve (AUC), which is calculated by taking the integral of the selected tracing over the duration of time for the selected tracing (mN x
min). Statistical analyses were performed using non-parametric tests as appropriate for the desired comparisons using GraphPad prism 9 software (GraphPad software). Individual tests are listed in the results section. Data were normalized to spontaneous contraction data or agonist-augmented data as appropriate. Data are reported as median (interquartile range, IQR) with \( P < 0.05 \) considered statistically significant.
CHAPTER IV
RESULTS

Evaluate the expression and distribution of KCa2.3 and IP3R channels in human myometrium

To qualitatively assess the location of KCa2.3 and IP3R channels within myometrial smooth muscle, thin sections of myometrium were probed for channel expression via immunofluorescence. KCa2.3 channel, stained in red, and IP3R channel, stained in green, were evaluated for myometrial expression and distribution, as well as changes due to pregnancy. Muscle fibers in the myometrium are oriented along different planes, therefore signal took on an elongated appearance when muscle fibers were cut obliquely and a rounded form when cut horizontally. Orientation of muscle fibers and tracts of signal were more apparent in non-pregnant tissue and corresponded to sweeping motions of channel expression viewed amongst truncated circular shapes (Figure 8). In pregnant tissue these distinct fibers were replaced with more segmental orientation and indistinct alignment of muscle fibers (Figure 10).

In non-pregnant myometrium KCa2.3 signal was generally diffuse with smaller punctated regions of signal. IP3R signal found similar characteristic to KCa2.3 expression but with larger densities of signal noted within the tissue (Figure 8). KCa2.3 signal was more variable than IP3R signal in non-pregnant tissue as some samples
demonstrated very distinct and clear expression (Figure 9A) and others were a more diffuse pattern of distribution (Figure 9B). IP3R channel expression and distribution generally remained the same across the assorted channel patterns. There was not a consistent ore representative staining pattern of KCa2.3 and IP3R within non-pregnant tissue as can be seen by non-pregnant sample images (Figure 9A and 9B). There were disparities noted in co-localization of channel signal as Figure 9A shows an expression pattern in which KCa2.3 and IP3R channels are distinctive with no co-expression and Figure 9B has a greater degree of co-expression. Immunofluorescent staining of multiple histological samples discovered an assortment of distribution and expression patterns within non-pregnant tissues. Glandular and stromal tissue were identified in non-pregnant myometrium and these tissues had different configurations than smooth muscle fibers with either KCa2.3 and nuclei or just IP3R but rarely co-localization (Figure 9B). The nuclear arrangement seen in non-pregnant tissue was dense and tight, with many smaller nuclei visualized in each image.

In pregnant myometrial samples cellular density decreased as uterine myocytes become hypertrophied, resulting in a nuclear staining pattern that is less condensed (Figure 10). IP3R channel distribution patterns remained generally consistent between non-pregnant and pregnant tissue samples, with slight variation in background dispersion (Figure 10). The greatest change observed between tissues of different gestational states was the expression and distribution patterns of KCa2.3 channels. KCa2.3 channels in pregnant tissue tended to be more diffusely distributed with varying intensities of expression (Figure 10). Generally, there was a greater loss of punctated and clustered regions of signal in pregnant tissue when compared to non-pregnant tissue.
Figure 8. KCa2.3 and IP3R channels are present in non-pregnant human myometrium. Individual channel signal was visualized with KCa2.3 channels stained red, IP3 channels stained green, and nuclei blue. A merged image of channel signals is presented in the far-right box. Areas of distinct signal were present as well as areas of potential co-expression seen in yellow.
Figure 9. Varying staining patterns of KCa2.3 and IP3R channels are visualized in non-pregnant tissue. Non-pregnant myometrium revealed an assortment of staining patterns for KCa2.3 and IP3R channels with some having distinct and punctated signal (A) and others with a more diffuse appearance of signal (B). Amount of co-expression also varied amongst samples.

Figure 10. KCa2.3 and IP3R channels are present in pregnant human myometrium. KCa2.3 channels are stained red, IP3 channels are stained green, and nuclei blue. A merged image of channel signals is presented in the far-right box. Areas of distinct signal were present as well as areas of potential co-expression seen in yellow.
Figure 11. Expression and distribution patterns of KCa2.3 and IP3R are variable in pregnant tissue. A range of staining patterns for KCa2.3 and IP3R channels were observed in pregnant tissue with a greater number of pregnant samples having a more diffuse KCa2.3 signal (A) than non-pregnant tissue. Patterns of co-localization of KCa2.3 and IP3R channels also varied within pregnant tissue (B).

Characterize spontaneous and augmented contractions of human uterine tissue

Reproductive state and spontaneous contraction characteristics

To characterize spontaneous contractions of non-pregnant and pregnant tissue, uterine rings were mounted around myograph pins and submersed in warmed (37°C) HEPES buffer. The rings were stretched until they held 1-4 mN of tone and allowed to rest until spontaneous contractions developed. Multiple uterine rings were sampled from the same patient to characterize aspects of individual subjects as well as the reproductive status as a group. Non-pregnant samples \(n = 109\) from 18 subjects and pregnant samples \(n = 159\) from 19 subjects were assessed for innate frequency and amplitude of contractions. Tissue samples generally developed spontaneous contractions in 1-3 hours.
but 6 of 156 (3.85%) non-pregnant samples and 41 of 216 (18.98%) pregnant samples did not develop spontaneous contractions after 3.5 hours.

Comparison of non-pregnant and pregnant spontaneous contractions began with plotting the frequencies (Figure 12) and amplitudes (Figure 13) of non-pregnant ($n = 109$) and pregnant ($n = 159$) samples. These graphs revealed observable differences in distributions and therefore histograms for frequency and amplitude were constructed.

![Figure 12](image.png)

**Figure 12. Frequencies of human uterine contractions in non-pregnant and pregnant tissue.** Individual samples are plotted to display the differences in clustering pattern between non-pregnant (A, $n = 109$) and pregnant (B, $n = 159$) tissue. Horizontal lines represent median and IQR.
Analysis demonstrated that pregnant tissue had a narrower window of spontaneous frequency as 75.5% of pregnant samples (n = 159) had a frequency less than 0.4, compared to 46.8% of non-pregnant samples (n = 109) having a frequency less than 0.4 (Figure 14). Amplitude histograms of non-pregnant and pregnant tissue reveal right skewed distributions but to a greater extent in pregnant tissue (Figure 15).

Figure 13. Amplitudes of human uterine contractions in non-pregnant and pregnant tissue. Individual samples are plotted to display the differences in clustering pattern between non-pregnant (A, n = 109) and pregnant (B, n = 159) tissue. Horizontal lines represent median and IQR.
Contraction frequency was significantly different between non-pregnant and pregnant tissue (0.53 [0.86-0.31] vs 0.26 [0.35-0.20], \( P < 0.0001 \)) which supports the theory that reproductive status alters innate contraction characteristics (Figure 16A). Amplitude of contractions was also different (Figure 16B), with pregnant tissue amplitude significantly reduced compared to non-pregnant samples (10 [16-6.2] vs 2.4 [6-0.96], \( P < 0.0001 \)). This finding supports the idea that pregnancy affects contractility, but the initial theory was that amplitude of pregnant contractions would be greater than non-pregnant contractions.

This trend of variation, noted in reproductive status comparison, was also observed between different samples from the same subject. Myographical recordings of spontaneous contractions showed noticeable variations of frequency, amplitude, and waveform morphology (Figure 17).
Figure 14. Histogram of spontaneous human uterine contraction frequencies. Frequency distribution of uterine contraction frequencies of non-pregnant (A) and pregnant (B) samples are skewed to the right. Frequencies of pregnant tissue is right skewed to a greater extent.

Figure 15. Histogram of spontaneous human uterine contraction amplitudes. Frequency distribution of uterine contraction amplitudes of non-pregnant (A) and pregnant (B) samples are skewed to the right. Amplitudes of pregnant tissue are skewed more to the right compared to non-pregnant contractions.
Figure 16. Comparison of frequencies and amplitude between non-pregnant and pregnant samples. Contraction frequency was significantly reduced in pregnant (n = 159) vs non-pregnant (n = 109) tissue samples. Contraction amplitude of pregnant tissue was also significantly less than non-pregnant tissue. Box plot of medians with min and max values represented by whisker bars. Data were analyzed by Mann-Whitney test. **** P < 0.0001.
Figure 17. Variation of contraction frequency and amplitude within same subject samples. Myographical recordings demonstrate different contraction frequencies, amplitudes, and waveform morphologies amongst tissue samples from the same pregnant subject.
To investigate the effects of KCa2.3 positive modulation via CyPPA on human uterine contractions, non-pregnant and pregnant uterine rings were mounted around myograph pins in an organ bath and allowed to develop spontaneous contractions via methods previously described. Increasing amounts of CyPPA (3 µM, 10 µM, 30 µM) were added to the organ bath in 20-minute increments. Myographical recordings demonstrate the changes in contraction frequency and amplitude with increasing CyPPA concentrations (Figure 18).

Individual sample response to increasing concentrations of CyPPA was inconsistent regarding frequency and amplitude changes. Figures 19 and 20 reveal these irregularities, as progression of frequency and amplitude over the course of CyPPA treatment in non-pregnant and pregnant tissue are compared side by side. Collected data revealed 30 µM CyPPA significantly reduced the frequency of spontaneous contractions in both the non-pregnant (0.28 [0.45-0.09] vs 0.83 [1.17-0.37], $P = 0.0001$; Figure 21A) and pregnant samples (0.14 [0.19-0] vs 0.27 [0.46-0.2], $P < 0.0001$; Figure 21B). There was also significant reduction of frequency between 3 µM and 30 µM CyPPA concentrations in the pregnant group (0.24 [0.41-0.17] vs 0.14 [0.19-0], $P = 0.002$; Figure 21B). Analysis of amplitude changes revealed a statistically significant decrease in uterine contraction amplitude in non-pregnant samples between 3 µM CyPPA and 30 µM CyPPA concentrations (13.9 [21.12-9.3] vs 8.51 [18.14-2.75], $P = 0.006$; Figure 22A). Pregnant sample amplitudes were also significantly reduced between spontaneous contractions and 30 µM CyPPA (2.97 [7.91-1.37] vs 0.5 [2.16-0], $P < 0.0001$) and
between 3 µM CyPPA and 30 µM CyPPA concentrations (2.54 [10.86-1.18] vs 0.5 [2.16-0], \( P < 0.0001 \); Figure 22B).

Figure 18. Effect of CyPPA on human uterine contractions in concentration-response studies. Myographical recordings demonstrating the effect of increasing CyPPA concentrations (3 µM, 10 µM, 30 µM) on spontaneously contracting uterine rings from non-pregnant (NP) and pregnant (P) human uterine tissue.
Figure 19. Effect of increasing CyPPA on human uterine contraction frequency of individual samples. Summary of contraction frequency changes of individual uterine rings with increasing CyPPA concentration from non-pregnant (A, $n = 8$) and pregnant (B, $n = 14$) human uterine tissue.

Figure 20. Effect of increasing CyPPA on human uterine contraction amplitude of individual samples. Summary of contraction amplitude changes of individual uterine rings with increasing CyPPA concentration from non-pregnant (A, $n = 8$) and pregnant (B, $n = 14$) human uterine tissue.
Figure 21. Summary of increasing CyPPA concentration on contraction frequency. Increasing concentrations of CyPPA reduced contraction frequencies in non-pregnant (A; \(n = 8\)) and pregnant (B; \(n = 14\)) tissue samples. Significant reductions in contraction frequency were found in non-pregnant and pregnant tissue samples. Box plot of medians with min and max values represented by whisker bars. Data were analyzed by Friedmann test with Dunn post hoc test for multiple comparisons. ** \(P \leq 0.01\), *** \(P \leq 0.001\), **** \(P < 0.0001\).
**Figure 22. Summary of increasing CyPPA concentration on contraction amplitude.**
The concentration-response displays a significant reduction of contraction amplitude with cumulative additions of CyPPA in non-pregnant (A; n = 8) and pregnant (B; n = 14) tissue samples. Box plot of medians with min and max values represented by whisker bars. Data were analyzed by Friedmann test with Dunn test for multiple comparisons. **$P \leq 0.01$, ****$P < 0.0001$.**
Contraction frequency and amplitude are components of the overall force generated by a contraction. Waveforms can vary in morphology leading to frequency and amplitude values lacking representation of contractility. To better assess overall changes to uterine contractions, shifts in AUC after increasing CyPPA concentrations were evaluated. Comparing changes in AUC, to frequency and amplitude, can infer the weight of these individual components to overall contractility. Graphical representation of changes in AUC with increasing CyPPA in non-pregnant and pregnant samples reveals similarities between non-pregnant and pregnant tissue with outliers (Figure 23).

Figure 23. Effect of increasing CyPPA on human uterine contraction AUC of individual samples. Summary of AUC changes of individual uterine rings with increasing CyPPA concentration from non-pregnant (A; n = 8) and pregnant (B; n = 14) human uterine tissue.
There was significant decrease in contraction AUC in non-pregnant samples between spontaneous contractions and 10 µM CyPPA (6296.5 [10546.75-5464] vs 4698 [7048.5-2902.75], \( P = 0.02 \)) and spontaneous contractions and 30 µM CyPPA concentrations (6296.5 [10546.75-5464] vs 2421 [4731.25-1287.5], \( P < 0.0001 \); Figure 24A). Pregnant tissue demonstrated a significant reduction in AUC between spontaneous contractions and 30 µM CyPPA (2409.5 [6068-756.03] vs 746 [2662.25-266.75], \( P < 0.0001 \)) and between 3 µM and 30 µM CyPPA concentrations (2002.5 [4611.75-612.7] vs 746 [2662.25-266.75], \( P < 0.0001 \); Figure 24B).

**Figure 24. Summary of increasing CyPPA concentration on contraction AUC.** The concentration-response displays a significant reduction of contraction AUC with cumulative additions of CyPPA in non-pregnant (A; \( n = 8 \)) and pregnant (B; \( n = 14 \)) tissue samples. Box plot of medians with min and max values represented by whisker bars. Data were analyzed by Friedmann test with Dunn test for multiple comparisons. \( \ast P < 0.05, \ast \ast \ast \ast P < 0.0001 \)
To compare the effect of CyPPA between non-pregnant and pregnant tissues, raw data needed to be normalized to spontaneous contractions within each gestational group. The normalized values of frequency, amplitude, and AUC after treatment with 30 µM CyPPA were compared between non-pregnant and pregnant tissue. Contraction frequencies ($P = 0.6875$; Figure 25A) and AUC ($P = 0.3828$; Figure 25C) were not statistically different between gestational groups, demonstrating CyPPA has equal effects on those contraction indices regardless of gestational status. CyPPA significantly reduced contraction amplitude between non-pregnant and pregnant samples (77.99 [99.21-12.85] vs 34.4 [51.43-0], $P = 0.016$; Figure 25B). These results indicate that CyPPA has greater effects on diminishing contraction amplitude in human pregnant samples, but not to the point that it significantly reduces overall contractility.

**Assess the role of the internal calcium stores in KCa2.3 modulation of human uterine contractility**

**Examine CyPPA attenuation of uterine contractions with oxytocin treatment**

To investigate the relationship between internal store release and the relaxing effects of KCa2.3 positive modulation, increasing OT concentrations (0.3 nM, 1 nM, 3 nM) were added every 20 minutes to pregnant tissue ($n = 13$) already incubated with 10 µM CyPPA. The hypothesis for this portion of the study is that increasing internal store calcium release, via OT and GPCR mediated mechanisms, will provided directed calcium to KCa2.3 channels for activation. As the calcium sensitivity of these channels is already
Figure 25. Comparison of non-pregnant and pregnant human myometrial samples after treatment with 30 µM of CyPPA. Treatment with 30 µM of CyPPA did not significantly affect contraction frequency (A) or AUC (C) between non-pregnant (n = 8) and pregnant samples (n = 14). Contraction amplitude (B) was significantly reduced in pregnant samples, displaying greater effect of CyPPA on pregnant tissue. Box plot of medians with minimum and maximum values represented by whisker bars. Data were analyzed by Wilcoxon test. * P < 0.05
increased though CyPPA positive modulation, amplification of internal store release
should further enhance uterine relaxation.

Inconsistent responsiveness to OT was noted in pregnant uterine tissue, as can be
demonstrated by plotting response to frequency, amplitude, and AUC of individual
samples (Figure 26). Initial incubation with CyPPA and then subsequent increasing
concentrations of OT did not yield significant changes to overall frequency ($P = 0.23$;
Figure 27A), amplitude ($P = 0.6$; Figure 27B), or AUC ($P = 0.16$; Figure 27C). While the
lack of significance does not support the internal stores hypothesis, the general trend of
OTs inability to overcome the relaxant effects of CyPPA was noted (Figure 27).
This same mechanism of proposed internal store amplification of KCa relaxant effects
was tested to assess if altering the addition order and concentration of OT and CyPPA
effected overall contractility. For this arm of the study, pregnant samples were incubated
with 1 nM OT for 20 minutes and then administered increasing dosages of CyPPA (3
µM, 10 µM, 30 µM) every 20 minutes. Variations in contractile response to the CyPPA
pretreatment protocol did not appear to be as numerous, but outliers, specifically when
analyzing changes in amplitude (Figure 28B), were identified when individual sample’s
contractile response were plotted (Figure 28).

Overall contractile effects of OT pretreatment can be seen in Figure 29. Analysis
demonstrated a significant decline in contraction frequency between 1 nM OT and 10 µM
CyPPA (0.313 [0.49-0.19] vs 0.15 [0.28-0.11], $P = 0.04$) as well as between 1 nM OT
and 30 µM CyPPA (0.313 [0.49-0.19] vs 0.067 [0.11-0.044], $P = 0.0005$; Figure 29A).
Amplitudes of contractions were greatly reduced by 30 µM CyPPA as significance was
demonstrated between it and all other aspects of the protocol:
Figure 26. Effect of CyPPA pretreatment protocol on pregnant human uterine contraction frequency, amplitude, and AUC of individual samples. Changes to frequency (A), amplitude (B), and (C) AUC of individual uterine samples from pregnant patients ($n = 13$). Line plots demonstrate a variable response to CyPPA and OT in pregnant tissue samples.
Figure 27. Effects of CyPPA and OT treatment on human myometrial contractility. Pregnant tissue samples \((n = 13)\) were incubated with 10 \(\mu\)M CyPPA with increasing concentrations of OT added to the myograph bath every 20 minutes after. Effects on frequency (A), amplitude (B), and AUC (C) of contractions were examined. Box plot of medians with min and max values represented by whisker bars. Data were analyzed by Friedmann test with Dunn test for multiple comparisons.
1 nM OT (1.71 [4.2-0.53] vs 0.83 [1.65-0.18], $P = 0.02$), 3 µM CyPPA (2.01 [4.65-0.58] vs 0.83 [1.65-0.18], $P = 0.0001$), and 10 µM CyPPA (1.81 [4.19-0.63] vs 0.83 [1.65-0.18], $P = 0.003$; Figure 29B). As would be expected from significant reductions in contraction frequency and amplitude, AUC exhibited statistical significance on a few levels. The AUC was lessened between 1 nM OT and 10 µM CyPPA (9883 [23962-353] vs 5803 [17750-2171], $P = 0.04$), 1 nM OT and 30 µM CyPPA (9883 [23962-353] vs 3798 [8498-1262.5], $P = 0.0003$), and 3 µM CyPPA and 30 µM CyPPA (8678 [23293-2849.5] vs 3798 [8498-1262.5], $P = 0.003$; Figure 29C).

The previous studies compared results among the same treatment group, which only found significance with OT pretreatment and increasing concentrations of CyPPA. Choosing what end points to compare posed two questions: (1) is there a difference if the same concentrations of agents are added but in different orders and (2) were the overall end point of the two treatment protocols significantly different in their ability to relax contractions. To compare the two groups, contraction traits (frequency, amplitude, and AUC) were normalized to spontaneous contractions (control). Figure 30A examines shifts in contractility (AUC) when the same concentration of agents, 10 µM CyPPA and 1 nM OT, are added in different orders. To assess the overall end points of each treatment protocol, contractility (AUC) at the end of each protocol were compared to spontaneous contractions. This meant that the concentration of agents at end point of the CyPPA pretreatment protocol was 10 µM CyPPA and 3 nM OT, and the OT pretreatment protocol was 1 nM OT and 30 µM CyPPA. These results can be found in Figure 30B.
Figure 28. Effect of OT pretreatment protocol on pregnant human uterine contraction frequency, amplitude, and AUC of individual samples. Changes to frequency (A), amplitude (B), and (C) AUC of individual uterine samples from pregnant subject (n = 13). Line plots reveal inconsistent responses to OT and CyPPA but with a trend of reduction in AUC.
Figure 29. Effects of OT and CyPPA treatment on human myometrial contractility. Pregnant tissue samples \((n = 13)\) were incubated with 1 nM OT with increasing concentrations of CyPPA added to the myograph bath every 20 minutes after. Effects on frequency (A), amplitude (B), and AUC (C) of contractions were examined. Box plot of medians with min and max values represented by whisker bars. Data were analyzed by Friedmann test with Dunn test for multiple comparisons. * \(P < 0.05\), ** \(P \leq 0.01\), *** \(P \leq 0.001\)
**Figure 30. Difference in AUC of contractions between two treatment protocol involving varying concentrations and application order of CyPPA and OT.** (A) Treatment protocols with concentrations of agents conserved but added to the organ bath in a different order (B) Treatment protocol assessing the end point of maximal concentrations of difference agents. Box plot of medians with min and max values represented by whisker bars. Data were analyzed by Wilcoxon test. * $P < 0.05$

**Alterations to CyPPA augmented contractions after internal store depletion**

To determine the ability of CyPPA to diminish uterine contractions in the presence of internal store depletion via SERCA blockade, non-pregnant and pregnant uterine rings were mounted and allowed to develop spontaneous contractions. Non-pregnant ($n = 9$) and pregnant ($n = 10$) contracting samples were incubated with 20 µM CPA (SERCA inhibitor) for 20 minutes or continue with control contractions, prior to the
addition of increasing amounts of CyPPA (3 µM, 10 µM, 30 µM) every 20 minutes.

Figure 27 portrays a representative myograph recording of the protocol and standard contractile effects on amplitude, frequency, and waveform morphology. A common observation of this protocol was an increase in contraction frequency after the addition of CPA which can be seen in Figure 31B.

Figure 31. Myographical recording of changes to contractions during execution of CPA and CyPPA protocol. Application of CPA was found to cause waveform changes in myometrial tissue samples. In this uterine sample a reduction of amplitude, an increase in frequency, and the development of tone was noted in the lower panel of this recording.
To compare the two treatment groups, contraction features were normalized to either spontaneous contractions in the control arm or to CPA modified contractions in the CPA and CyPPA arm. Normalizing the data controlled for the CPA induced contractile changes, allowing for comparison of CyPPA effects between the groups. Normalized values of frequency, amplitude and AUC are presented in Figure 32. Normalized frequency results for non-pregnant samples were not significant between the control and CPA treatment group, with calculated $P$ values of 0.65 for 3 $\mu$M CyPPA, 0.91 for 10 $\mu$M CyPPA, and $> 0.99$ for 30 $\mu$M CyPPA (Figure 32A). Non-pregnant contraction amplitude comparison between control and CPA pretreated groups were not statistically different at 3 $\mu$M CyPPA ($P > 0.99$), 10 $\mu$M CyPPA ($P > 0.99$), or 30 $\mu$M CyPPA ($P = 0.11$) (Figure 32C). Overall contractility, in the form of normalized AUC for non-pregnant tissue, did not support significance and included $P = 0.57$ for 3 $\mu$M CyPPA, $P = 0.82$ for 10 $\mu$M CyPPA, and $P = 0.38$ for 30 $\mu$M CyPPA (Figure 32E).

Pregnant myometrial sample data showed no difference in contraction frequency between control and treatment groups with $P$ values of 0.23 for 3 $\mu$M CyPPA, 0.49 for 10 $\mu$M CyPPA, and 0.32 for 30 $\mu$M CyPPA (Figure 32B). Contraction amplitude comparison between pregnant control and CPA pretreated groups did not demonstrate significance at 3 $\mu$M CyPPA ($P = 0.85$), 10 $\mu$M CyPPA ($P > 0.99$), or 30 $\mu$M CyPPA ($P = 0.56$) (Figure 32D). Overall contractility, in the form of normalized AUC in pregnant samples, included $P = 0.92$ for 3 $\mu$M CyPPA, $P = 0.56$ for 10 $\mu$M CyPPA, and $P = 0.23$ for 30 $\mu$M CyPPA (Figure 32F).

Blocking SERCA in myometrial smooth muscle allowed us to remove the internal calcium stores and test its role in uterine contractions. We found depleting SR stores did
not hamper CyPPA’s ability to diminish uterine contractions, suggesting that in an unstimulated state, internal stores are not a main contributor to SK3 channel activation.

**Characterize effects of increased internal calcium release on spontaneous and KCa2.3 modulated contractions by GPCR and PLC agents**

To initially characterize the effects of internal calcium store release on spontaneous contractions, GPCR and PLC pathways were augmented through different agents. GPCR agonists included oxytocin and PGF2a, both of which activate IP3R channels through increased formation of IP3 as one of their mechanisms. Stimulation of the PLC pathway also increases IP3 generation and internal store release, and was activated via m-3M3FBS, and inhibited with U73122. Pregnant uterine samples \( n = 4 \) were allowed to develop spontaneous contractions, with changes to contraction characteristics monitored after the addition of either 1 nM OT, 0.1 µM PGF2a, 30 µM m-3M3FBS, or 10 µM U73122 to the organ bath.

The effect on contraction frequency after addition of GPCR and PLCs agents was variable (Figure 33A). No significant effects on frequency were found by addition of OT \( (P = 0.875) \), PGF2a \( (P = 0.375) \), m-3M3FBS \( (P = 0.875) \), or U73122 \( (P = 0.5) \). Analysis of amplitude changes demonstrated no significance with agents having the following values: OT \( (P = 0.25) \), PGF2a \( (P = 0.375) \), m-3M3FBS \( (P = 0.375) \), U73122 \( (P = 0.5) \). While not statistically significant, agents that increased internal calcium store release did show a trend in increasing contraction amplitudes, as was expected due to increased cytosolic calcium levels from multiple mechanisms (Figure 33B). Overall cumulative
Figure 32. Summary of contractile results in the comparison of increasing CyPPA concentration with or without CPA pretreatment. Control and CPA treated non-pregnant (n = 9) and pregnant (n = 10) uterine samples were compared for changes in contraction frequency (A, B), amplitude (C, D), and AUC (E, F). Data were normalized to either control contractions or CPA stimulated contractions for comparison between the treatment groups. Box plot of medians with minimum and maximum values represented by whisker bars. Data were analyzed by Wilcoxon matched-pairs rank test with false discovery rate approach for multiple comparisons.
changes in contractions, in the form of AUC, were not found to be significant for 
OT ($P = 0.625$), PGF2a ($P = 0.125$), m-3M3FBS ($P > 0.99$), or U73122 ($P > 0.99$; Figure 33C).

Identifying baseline contraction changes by each GPCR or PLC agent allowed us to assess further alterations by positive or negative KCa2.3 modulators. Spontaneously contracting tissue were incubated with either 10 µM CyPPA ($n = 9$) or 0.5 µM apamin ($n = 9$) for 20 minutes, with the addition of 1 nM OT, 0.1 µM PGF2a, 30 µM m-3M3FBS, or 10 µM U73122 afterwards. Results of these studies continued to demonstrate a variable response of pregnant tissue to CyPPA.

Myographical examples of these findings (Figure 34) show uterine samples from the same pregnant patient having inconsistent response to 10 µM CyPPA. Both CyPPA and apamin, in the presence of a GPCR or PLC agent, were compared to that agent’s baseline functioning to assess the ability of KCa2.3 channel modulation to alter contractions. Changes in frequency were analyzed and found to not be significant with OT augmented contractions compared to CyPPA with OT ($P > 0.99$) and apamin with OT ($P > 0.99$). Amplitude assessment of OT vs CyPPA with OT ($P = 0.41$) and OT vs apamin with OT ($P = 0.66$) did not demonstrate significance and AUC results of OT vs CyPPA with OT ($P = 0.5$) and OT vs apamin with OT ($P > 0.99$) were not significant either (Figure 35).

CyPPA and apamin were not able to significantly alter PGF2a contraction baseline frequency as $P$ was > 0.99 for both PGF2a vs CyPPA with PGF2a and PGF2a vs apamin with PGF2a. Baseline contraction amplitudes were also not significantly altered.
Figure 33. Alterations to spontaneous contractions of pregnant uterine tissue after administration of GPCR or PLC agents. Graphical representation of changes in contraction frequency (A), amplitude (B), and AUC (C) of pregnant uterine sample (n = 4) after the administration of either 1 nM OT, 0.1 µM PGF2α, 30 µM m-3M3FBS, or 10 µM U73122. Box plot of medians with minimum and maximum values represented by whisker bars. Data were analyzed by Wilcoxon signed rank test.
Figure 34. Myographical recordings of pregnant tissue samples from the same subject incubated with CyPPA prior to administration of GPCR or PLC agents. The uterine tissue sample in panel A has no observed response to 10 µM CyPPA, while the waveform of the sample in panel B undergoes a morphological change. The sample in Panel D has a slight increase in frequency and reduction of amplitude.
Figure 35. Effects of oxytocin on pregnant uterine contractions in presence of KCa2.3 channel modulators. Pregnant uterine samples \( (n = 9) \) pretreated with OT (1 nM) were exposed to KCa2.3 channel positive modulator, CyPPA (10 µM) or KCa2.3 channel negative modulator, apamin (0.5 µM). Alterations to contraction frequency (A), amplitude (B), and AUC (C) were analyzed. Box plot of medians with minimum and maximum values represented by whisker bars. Data were analyzed by Kruskal-Wallis test.
Figure 36. Effects of prostaglandin F2α on pregnant uterine contractions in presence of KCa2.3 channel modulators. Pregnant uterine samples (n = 9) pretreated with PGF2α (0.1 µM) were exposed to KCa2.3 channel positive modulator, CyPPA (10 µM) or KCa2.3 channel negative modulator, apamin (0.5 µM). Alterations to contraction frequency (A), amplitude (B), and AUC (C) were analyzed. Box plot of medians with minimum and maximum values represented by whisker bars. Data were analyzed by Kruskal-Wallis test.
Figure 37. Effects of m-3M3FBS on pregnant uterine contractions in presence of KCa2.3 channel modulators. Pregnant uterine samples ($n = 9$) pretreated with m-3M3FBS (30 µM) were exposed to KCa2.3 channel positive modulator, CyPPA (10 µM) or KCa2.3 channel negative modulator, apamin (0.5 µM). Alterations to contraction frequency (A), amplitude (B), and AUC (C) were analyzed. Box plot of medians with minimum and maximum values represented by whisker bars. Data were analyzed by Kruskal-Wallis test.
as PGF2a vs CyPPA with PGF2a incurred a $P > 0.99$ and PGF2a vs apamin with PGF2a ended with a $P = 0.99$. Collective assessment of contractility via AUC values revealed neither PGF2a vs CyPPA with PGF2a ($P = 0.98$) or PGF2a vs apamin with PGF2a ($P = 0.22$) meeting statistical significance (Figure 36).

Outcomes of KCa2.3 channel modulation were assessed with the PLC activator m-3M3FBS. Frequency results of m-3M3FBS vs CyPPA with m-3M3FBS ($P = 0.38$) and m-3M3FBS vs apamin with m-3M3FBS ($P = 0.71$) proved to not be significant, as was the case for amplitude results: m-3M3FBS vs CyPPA with m-3M3FBS ($P = 0.78$), m-3M3FBS vs apamin with m-3M3FBS ($P > 0.99$). Neither KCa modulator was able to significantly alter the AUC baseline, with m-3M3FBS vs CyPPA with m-3M3FBS earning a $P = 0.7824$, and m-3M3FBS vs apamin with m-3M3FBS at a $P = 0.88$ (Figure 37).

U73122 was the lone antagonist to internal calcium store release tested in this arm of the project. As a decrease of internal store release would reduce the activation of the KCa2.3 negative feedback system, it was postulated U73122 would increase contractility in the presence of CyPPA. When the KCa2.3 hyperpolarizing effect is inhibited via apamin, decreased store calcium release was theorized to reduce contractility. CyPPA with U73122 did not significantly alter frequency ($P > 0.990$, amplitude ($P > 0.99$), or AUC ($P > 0.99$) when compared to U73122 alone. This lack of significance was also true for apamin, as apamin with U73122 was unable to significantly change frequency ($P = 0.52$), amplitude ($P > 0.99$), or AUC ($P = 0.78$), compared to U73122 augmented contractions (Figure 38).
Figure 38. Effects of U73122 on pregnant uterine contractions in presence of KCa2.3 channel modulators. Pregnant uterine samples \((n = 9)\) pretreated with U73122 (10 µM) were exposed to KCa2.3 channel positive modulator, CyPPA (10 µM) or KCa2.3 channel negative modulator, apamin (0.5 µM). Alterations to contraction frequency (A), amplitude (B), and AUC (C) were analyzed. Box plot of medians with minimum and maximum values represented by whisker bars. Data were analyzed by Kruskal-Wallis test.
Apamin as a pro-contractile agent was not supported through statistical analysis, or graphical representation, as myographical representations of apamin effects on contractions ranged from observed minimal amplification to relaxation (Figure 39).

**Figure 39.** Myographical recordings of pregnant tissue incubated with apamin prior to administration of GPCR or PLC agents. Apamin, which recovers CyPPA augmented contractions through KCa2.3 pore blocking effects, showed variable effects on pregnant tissue. Reductions in amplitudes were observed in panels A and C, with frequency decreasing in panel A, B, and D, and increasing in panel C.
CHAPTER V
DISCUSSION

Myometrial studies in mouse, rat, and human tissues have proposed a role of KCa2.3 channels in the negative feedback control of uterine contractions. The purpose of this project was to assess the impacts of different sources of calcium sources for activation of KCa2.3 channels. This was performed by characterizing the effects of internal calcium store modulation on contractions and evaluating the potential relationship between internal calcium stores and KCa2.3 channels. To achieve this, it was necessary to characterize spontaneous contractions of non-pregnant and pregnant human uterine tissue, determine the ability of CyPPA to inhibit gestationally different human uterine contractions, and examine CyPPA efficacy in the presence of amplified and diminished internal calcium store release.

KCa2.3 channels were first identified as a potential regulator in myometrial contractibility when Bond et al. observed that mice who conditionally overexpressed KCa2.3 channels (SK3T/T) had protracted delivery.12 Further studies in mice by Brown et al. identified that the KCa2.3 channels were present in myometrial tissue21 and Pierce et al. reported KCa2.3 channel expression in whole uterine samples decreased over the course of gestation22 with Skarra et al. recognizing a gestational decrease in murine KCa2.3 channel expression in myometrial samples.27 Noble et al. determined KCa2.3
channel expression was not statistically different between non-pregnant and pregnant samples, which provides some evidence of species dependent channel differences. Human uterine assessment by Mazzone et al. revealed a down-regulation of KCa2.3 transcript in pregnant myometrium versus non-pregnant myometrium. Rahbek et al. confirmed that KCa2.3 mRNA and protein levels were significantly lower in the myometrium from pregnant women compared to the non-pregnant group. This trend was observed both in the preterm and term group, as well as non-laboring and laboring group.

**Expression and distribution of KCa2.3 and IP3R channels in human myometrium**

Immunofluorescent studies with mouse myometrial samples by Skarra et al. found that KCa2.3 channels were highly concentrated along the cell periphery, consistent with predominant membrane localization and no obvious changes in cellular distribution over the course of gestation were visualized. Previous double immunofluorescent labeling experiments in human uterine tissue used KCa2.3 and CD34 (smooth muscle) antibodies. These studies found KCa2.3 expression in telocytes, which are interstitial Cajal-like cells, of non-pregnant human myometrium but not in smooth muscle cells. In pregnant human myometrium, staining for KCa2.3 channels was not present in smooth muscle cells or telocytes. No further information or gestational changes regarding KCa2.3 location or distribution were discussed.

Our findings differed from the previous study in that we found KCa2.3 expression throughout the myometrium with signal appearing to be in the muscle fibers due to the orientation of the signal and location relative to nuclei. It was difficult to visually
compare KCa2.3 expression levels between non-pregnant and pregnant as the signal was present throughout both tissue groups. More interestingly to discuss was the assortment of distribution patterns observed in the tissue; with all the variety it was not hard to speculate that tissues would respond differently. Cellular architecture is a concept that explains channel function through the components of channel expression and cellular distribution. Protein expression studies provide information on overall channel protein levels but does not answer if the channel is functional. A study could find that channel expression is high, but those channels could be in vesicles and not be in a position to have an effect. Specifically, with KCa channels, their proximity to a calcium source for activation has more functional value than their population. A few well-placed KCa channels next to a preferential source of calcium (VGCC, IP3R, TRP) could create enough repolarizing influence to trigger relaxation. On the opposite end, many KCa channels in an area without a calcium source channel requires the channels to rely on global calcium levels potential leading to a reduced repolarizing effect. The variability of channel arrangements, paired with the variability of tissue function, supports the concept that the cellular functional architecture determines the contractile properties of the tissue.

As KCa channels are often located near calcium sources we assessed the potential for co-localization of KCa2.3 and IP3R channels with dual immunofluorescent staining. Some samples showed very distinct individual channel signal with little co-expression, while other samples displayed small puncta or larger tracts of co-expression. Similar patterns of punctated clusters and densities were found with immunofluorescent studies of IP3R channels in swine coronary arterial endothelium. All the functional inconsistencies found in this study supports this variety of proximity staining and
warrants further investigation into this potential coupling with higher power magnification studies and proximity assessments.

The primary KCa2.3 antibody used in the dual staining protocol is one our laboratory has vetted and used in a variety of tissues, but the IP3R primary antibody is newer to our laboratory. Dual staining is known to have its difficulties and early in this project only rabbit primary antibodies were available. Dual staining with two rabbit primary antibodies resulted in a large amount of costaining where individual channels were not able to be distinguished. Primary antibodies from different species became available and allowed us to see distinct signals with lower amounts of costaining. We also tested our dual staining protocol on other tissues and while these antibodies are still being optimized, dual staining studies are resulting in clearer images.

**Spontaneous and augmented contractions of human uterine tissue**

**Characteristics of spontaneous contractions**

Few studies were found in the literature characterizing spontaneous contractions amongst non-pregnant and pregnant human subjects, with most having lower $n$ values. To better understand the start-point for performed studies, characterization of spontaneous contractions was necessary. Variation in frequency and amplitude of spontaneous contractions were observed, which created a vast scatter and spread of results. Age-related studies by Arrowsmith et al. demonstrated that AUC of non-pregnant and pregnant spontaneous contractions had much scatter as well. Further data subset analysis by their group controlled for parity, gestational age, mother’s BMI, baby’s birthweight,
and only a previous cesarean section as an indication for surgery, did not change the data spread.\textsuperscript{165} This study did not compare contraction characteristics between non-pregnant and pregnant tissue directly.

Arrowsmith \textit{et al.} evaluated pregnant spontaneous contractions of term and postdate women (total $n = 58$) and found mean contraction frequency to be 1.14 for term women and 1.87 for postdate women. Mean contraction amplitude was 3.6 mN for term pregnant women and 2.07 mN for postdate pregnant women.\textsuperscript{166} These values correlate with our findings for frequency and amplitude of pregnant contractions. Rosenbaum \textit{et al.} compared human myometrial contractions of non-pregnant and pregnant women and found amplitude, time between contractions, and AUC were significantly greater in pregnant samples.\textsuperscript{167} Raw data from their study is very different to what we collected, as their mean amplitude of non-pregnant tissue was 6.53 mN and 11.33 mN for pregnant tissue. Study differences include our use of uterine rings rather than strips and sample size. We used replicate samples, 109 samples from 18 non-pregnant women and 159 samples from 19 pregnant women. Their $n$ of 9 referred to the number of patients and it was assumed this was the total number of samples as they did not list otherwise. Additional study methods were similar and reasons for the difference is unclear.

The variation of contractile features and myographical waveforms of separate samples from the same patient, could be explained by disruption of the electrochemical syncytium. The method for preparation of myometrial samples results in samples that are not structurally uniform as amount of contractile tissue and orientation of muscle fibers between samples is not always the same. Immunofluorescence of myometrial samples identified distinct smooth muscle bands and confirmed their varied orientation. Should
the mean vector of force for a sample not align with the transducer pins, quantities of each contraction are not accounted for. One study found only 65% of the cross-sectional area of myometrial strips is contractile tissue\textsuperscript{168} and another study suggested 85-90% of uterine myocytes are oriented perpendicular to the axis of the strip.\textsuperscript{169} It is most likely that through sample dissection the orientation of muscle fibers and force vectors were compromised, leading to greater variability in spontaneous contractions. In order to create structurally uniform samples, we should aim to capture muscle fibers traveling in an orientation that is the same amongst samples and fully assessed by equipment, which is likely difficult. The force vector of non-pregnant tissue could be inferred due to the orientation of fibers, but unlikely in pregnant samples as the fibers became increasingly interwoven. Even with the identification of the force vector for an individual sample, the mean vector for the organ itself cannot be inferred due to the small section of tissue studied. Sampling of a small section of myometrial tissue was not reflective of the overall coordinative effects of the organ, especially in pregnant samples.

Rather than one pacemaker cell being dominant for a given region of tissue, dissection of the tissue into smaller strips causes new pacemakers to control the rate of action potential firing. When individual strips are regulated by different pacemakers, a variety of waveforms with variable characteristics are generated. Samples from pregnant patients had an increased rate of not developing contractions when compared to non-pregnant samples (18.98% vs 3.85%). As myocytes hypertrophy during pregnancy, using the same sample size between gestational groups may have led to a pacemaker cell not being captured in samples.
Based on gestational changes such as downregulation of KCa2.3 channels, increased gap junction formation, and upregulation of contraction-associated proteins, it was theorized that contraction amplitude would be greater in pregnant samples. Overall pregnant samples had smaller values for frequency and amplitude compared to non-pregnant samples, which was surprising. The frequency and amplitude distribution between gestational groups was also unanticipated, as we expected the shape of the histogram to remain generally similar, even if median values shifted. Instead, the histograms in pregnant samples were more right skewed than non-pregnant samples for contraction indices.

Smaller values for frequency and amplitude could be explained by the overall objective of a pregnant uterus. During labor the uterus does not attempt to create the largest contraction possible, but rather to coordinate myocyte activity into organ level involvement and provide deliberate movement of the fetus. Increased gap junctions and membrane potential reduction are two gestational adaptations with the goal of triggering an action potential that will propagate and recruit all uterine regions in a top-down fashion for effective delivery of the fetus. Based on this goal for contraction synchronization rather than magnitude, smaller amplitudes may be an appropriate and physiologic change during labor. Additional reasons for gestational differences could be due to maternal age as other groups found that older pregnant mothers had decreased contraction amplitude when compared to younger pregnant mothers. Retrospective analysis with segregation of laboring vs non-laboring and maternal age, may demonstrate a trend in spontaneous contractions between subgroups.
Myometrial contractile proteins may be another potential reason for lower amplitudes in pregnant women. During pregnancy there is increased expression of smooth muscle myosin, but due to increase in myocyte cell size, the amount of myosin per milligram of protein is maintained compared to a non-pregnant state.\textsuperscript{169} Caldesmon, a smooth muscle thin-filament-associated protein that inhibits generation of force by myosin, increases 4-fold in pregnant myometrium. A disproportionate increase in caldesmon expression relative to myosin during pregnancy may cause an inhibition of contractile force prior to initiation of labor.\textsuperscript{170} Most of the pregnant study participants were non-laboring and therefore the activation of contraction-associated proteins and physiologic amplification of contractions may not have occurred.

**CyPPA diminishes contractions in non-pregnant and pregnant human myometrium**

Skarra et al. identified that increasing concentrations of CyPPA did not have a significant effect on spontaneous contraction frequency in non-pregnant, mid-pregnant and term pregnant mice.\textsuperscript{27} Amplitudes for these contractions were suppressed in a dose-dependent fashion and could be recovered by apamin. CyPPA responses were also right shifted in mid- and term pregnancies compared to non-pregnant mouse samples, indicating reduced CyPPA sensitivity with pregnancy.\textsuperscript{27}

The only known human myometrial study utilizing CyPPA was conducted by Rosenbaum et al. who evaluated cumulative CyPPA concentrations (0.1 – 60 uM) in non-pregnant ($n = 6$) and pregnant ($n = 6$) patient samples. A concentration-dependent reduction of contractility was recorded, with 60 µM of CyPPA completely abolishing contractions. They assessed percentage of relaxation as a function of the integral (AUC) and did not evaluate frequency or amplitude changes.\textsuperscript{26}
This study is the first to assess individual contributions to contractions by evaluating frequency and amplitude, as components of overall contractility. We observed that individual non-pregnant and pregnant samples had variable responses to CyPPA. After plotting the progression of each sample through increasing concentrations of CyPPA it was noted that some samples had increases in frequency or amplitude over the treatment course. Other samples seemed to be unresponsive to CyPPA. Despite these inconsistencies we found significant reductions in contraction frequency, amplitude, and AUC through the course of treatment. Generally, all contractile components were reduced after 30 \( \mu \text{M} \) CyPPA (highest concentration assessed), with varying significance amongst other concentration interactions. We are the first to compare CyPPA effectiveness between non-pregnant and pregnant human myometrial samples. CyPPA demonstrated similar effects on contraction frequency and AUC between the gestational groups and CyPPA reduced contraction amplitude in the pregnant samples more than the non-pregnant samples. These results indicate that CyPPA has greater effects on diminishing contraction amplitude in human pregnant samples, but not to the point that it significantly reduces overall contractility.

Reasons for variability amongst pregnant tissue samples response to CyPPA could be due to differences in KCa2.3 location and distribution. Previous studies looked at KCa2.3 channel expression values and found human uterine channel levels decrease in late gestation.\(^{20}\) These studies only look at the protein expression and not the location or distribution patterns, which we evaluated. Immunofluorescent staining of non-pregnant and pregnant tissue samples demonstrated multiple patterns of KCa2.3 expression and distribution. Some samples had a very diffuse pattern of KCa2.3 staining while others
had regions of pronounced puncta or clustered densities. The assortment of immunofluorescent patterns would prompt me to theorize CyPPA would have varied effects. Differences in the functional architecture could alter the CyPPA response of a given tissue. The number of channels may not be as important as a channel’s proximity to a calcium source. A few KCa2.3 channels clustered around a VGCC or IP3R channel can preferentially be activated thereby increasing channel kinetics compared to widely distributed KCa2.3 channels without a close source of activation calcium. Samples with a stronger response to CyPPA could have one type of KCa2.3 expression pattern while samples that appeared unresponsive may have another type of channel expression pattern.

Gestational age of pregnant tissue could lead to inconsistent CyPPA response amongst tissue samples. Term pregnant samples were the largest sub-group within pregnant samples and their gestational age range was approximately four weeks. Parturition based hormone levels can begin to increase or decrease within a week, which may alter KCa2.3 expression levels and potentially distribution patterns. Progesterone has not been found to alter KCa2.3 expression\textsuperscript{19} but estrogen has. Two specificity protein transcription factors that are expressed in the uterus compete to regulate KCa2.3 channel expression during pregnancy in response to stimulation by estrogen.\textsuperscript{171} Week or more differences in gestational age could lead to altered KCa2.3 expression and therefore CyPPA response.

Non-pregnant samples were excised from uteri that were being removed due to confounding clinical manifestations. All the non-pregnant samples had a diseased state that could be altering multiple contractile properties. Care was taken to not sample from grossly abnormal tissue, but changes that were not observed via stereomicroscope are
likely. KCa2.3 expression patterns in diseased uterine tissue has not been studied, but
erine fibroids and endometriosis can alter expression of varying contractile proteins and
lead to changes in contractility if these samples were hypothetically compared to non-
diseased non-pregnant tissue.

Utilizing already obtained data, further studies can correlate functional data to
expression and distribution patterns to evaluate for potential trends. By comparing the
type of channels staining pattern to tissue CyPPA response we can investigate if
CyPPA’s effect depends on KCa2.3 distribution. Being able to predict expression pattern
based on functional data or vice versa, could allow future studies to sub-type tissue to
better compare similar samples. Correlation studies can also extend into the clinical
patient history. Detecting pairings of clinical features, such as underlying uterine
pathology, gestational age, maternal age, and number of previous deliveries, and
functional or imaging characteristics could allow investigators to stratify patients into
appropriate groups for comparisons. Additionally continuing to collect pregnant tissue in
attempts to increase the number of preterm delivery samples could evaluate CyPPA
effectiveness between preterm and term pregnancies to further strengthen evidence of
CyPPA as a potential tocolytic.

**Internal calcium stores and KCa2.3 modulation of human myometrial contractility**

**Human myometrial contractility after CyPPA and oxytocin treatment**

Oxytocin receptors increase during late gestations allowing serum oxytocin levels
to amplify contractions during labor. For a tocolytic medication to be effective it must be
able to maintain relaxation effects in the presence of physiologic contractile agents, therefore effects of contractions in the presence of oxytocin and CyPPA were evaluated. Skarra et al. found that application of CyPPA caused concentration-dependent decreases in contraction amplitude of non-pregnant and pregnant murine samples. Pretreatment with oxytocin did not significantly reduce CyPPA maximal effect in non-pregnant or pregnant samples, with CyPPA sensitivity reduced in pregnant samples.\textsuperscript{27}

In this study oxytocin administration led to tissue sample waveform changes that altered contraction frequency, amplitude, and sometimes both. Due to oxytocin’s multiple mechanisms, these waveform shifts were expected. Oxytocin increases calcium influx through membrane channels, triggers internal calcium store release through IP3 generation, and reduces the efflux of calcium from the cytoplasm, which can all lead to contractile changes. We expected some variability in sample response to oxytocin, based on clinical findings of inconsistent patient responses to oxytocin when it was administered for the initiation/stimulation of labor, but the degree of variability was unanticipated.\textsuperscript{172–174} Multiple calcium-based pathways are affected by oxytocin binding, creating an interdependent network of signals that can play a role in regulating contractions. Altering the functionality, expression levels, or location of OTRs can affect channel kinetics of membrane potassium channels and IP3R therefore modifying downstream effects. We did not stain for OTR expression or distribution in human myometrium, which should be included in a follow up project, but based on the variance of KCa2.3, and to a lesser extent IP3R, we can postulate that OTR distribution will also be variable. In deciphering a sample’s response to oxytocin, we looked for a waveform change as some samples seemed unresponsive to oxytocin, and if a waveform change
occurred what aspect of contractions changed; the frequency, amplitude, or both. As relationships between cellular elements are being assessed the inherent variability within each element’s arrangement in the cell and then their organization in relation to one another, creates greater potential for functional difference. Samples displayed an array of functional changes to oxytocin which we theorize is due to the underlying cellular architecture determining the specificity of response.

As previously discussed, the stage of pregnancy or labor may be affecting cellular architecture and response to agents due to shifts in gestational hormones. Preterm and term patients in a non-laboring situation may be in a transitional or transformative stage where functional relationships required for contraction coordination and synchronization have not matured. Estrogen levels regulate OTR expression levels which could result in non-laboring tissue having fewer functional OTRs and a reduced IP3R activation. Previous deliveries or underlying maternal conditions could also have an impact on tissue response to oxytocin through previous changes to uterine structure or chronic inflammatory conditions. Correlation studies comparing laboring tissue to non-laboring tissue in oxytocin response and KCa2.3 distribution patterns may uncover a pattern of response consistent with gestational age or laboring status. Further studies expanding on this idea and incorporating OTR expression and distribution analysis via immunofluorescence, could provide more information on the ability of cellular arrangement to effect tissue function.

Oxytocin was employed to investigate the relationship between increased internal store release and the relaxing effects of KCa2.3 positive modulation. We investigated what oxytocin did in the presence of CyPPA, whether oxytocin boosted contractility
through increased cytosolic calcium mechanisms or if CyPPA prevented oxytocin’s pro-contractile mechanisms. There was also the potential for preferential activation of KCa2.3 channels by IP3R-mediated calcium release, which has been found between IP3R and TRP channels, BKCa channels, and IKCa channels. In this portion of the project, we found that oxytocin was not able to overcome contractions previously diminished by CyPPA. CyPPA could still significantly reduce contractility in the presence of oxytocin, and the order in which these agents were added did not alter outcomes. These findings support that amplifying the innate KCa2.3 negative feedback mechanism with CyPPA, creates an environment where oxytocin’s effects are truncated. Increasing the calcium sensitivity of KCa2.3 turns on repolarization effects at lower cytosolic calcium levels and shuts down VGCC earlier in a contraction cycle. Decreasing the open time of VGCC reduces calcium influx and intracellular calcium levels, limiting the potential for activation of contractile machinery. Based on our findings, the pro-contractile mechanisms of oxytocin are not enough to overcome these effects. In some samples there was a greater reduction in contractility with increasing concentrations of oxytocin suggesting that in tissue with a certain structural arrangement, oxytocin may promote the relaxing effects of CyPPA by promoting release of internal calcium stores in close proximity to KCa2.3 channels, further amplifying the feedback response.

Additional studies are needed to evaluate the staining patterns of OTRs in tissue and potentially location relative to other channels to determine if this functional architecture is at the heart of these findings.
Alterations to CyPPA augmented contractions after internal store depletion

To determine if CyPA is still effective with internal stores depleted, we assessed contraction changes after SERCA blockade in non-pregnant and pregnant uterine samples. Addition of CPA led to changes in waveform morphology in both gestational groups. These changes were not always consistent but commonly there was an increase in frequency, and reduction in amplitude and development of some baseline tone. Tone is not associated with contractions per se, but rather intracellular calcium concentrations. Initially after addition of CPA and inhibition of the SERCA pump, there is an increase in global calcium levels, potentially explaining the development of tone and waveform changes. As no calcium reuptake is occurring, store operated channels sense low store levels and open for calcium influx, further increasing global calcium levels. Eventually the increased calcium will leave the cell via efflux channels, cytosolic calcium levels will return to normal and internal stores will be depleted. Any contraction after this point will be without the assistance of internal stores. Additional studies quantifying the tone created by CPA could shed further light on the internal store contributions.

Depleting the internal stores did not hamper CyPPAs ability to dimmish uterine contractions, suggesting that in an unstimulated state, internal stores are not a main contributor to KCa2.3 activation. CyPPAs capabilities in a stimulated state via prostaglandins or oxytocin could be evaluated in the future to further categorize the role of the internal stores in the KCa2.3 negative feedback mechanism.

CPA was chosen for its ability to deplete internal stores, but the initial goal of this project section was to block the IP3R channel. Unfortunately, because available pharmacologic inhibitors and modulators of IP3Rs are notoriously non-selective with
many off target effects, we were not able to precisely address IP3R functional role. Since we could not block the IP3R directly, we had to adjust the function of IP3R by controlling up and down stream regulators. Additional studies using siRNA, genetic or antibody approaches could be used to directly assess IP3 effects of KCa2.3 feedback.

**Effects of increased internal calcium release on spontaneous and KCa2.3 modulated human myometrial contractions**

Continuing with the hypothesis that increased internal store release would preferentially activate KCa2.3 channels and amplify the channels negative feedback mechanism, multiple agents that modified IP3 generation were assessed. These results were also an opportunity to assess two new agents, m-3M3FBS and U73122, that have never been studied in human myometrial tissue. First an assessment of the baseline effects these agents (OT, PGF2a, m-3M3FBS, U73122) have on spontaneous contractions were performed. There were no significant findings for this section of the study, but this section had the smallest sample size (n = 4). Due to the variability of contractions noted throughout this entire project, a n of 4 may not have been enough to gain significance. Future studies could continue to evaluate these new agents in a larger scale format. Of note Padal et al. evaluated m-3M3FBS in a high cholesterol pregnant murine model to demonstrate any effect on high cholesterol diet on the phospholipase C pathway. m-3M3FBS was added cumulatively (0 – 50 µM) to mouse uterine strips and it produced a concentration-related relaxation that was not statistically significant.175

Once expected alterations to contractions by the GPCR and PLC agents were established, additional studies looked at potential deviation from baseline with positive or negative modulation of KCa2.3 channels. Results of these studies continued to
demonstrate a variable response of pregnant tissue to CyPPA and apamin. While not significant, CyPPA’s trend of relaxation compared to internal store agonists baseline, did align with initial hypotheses, if not due to cooperation between internal stores and KCa2.3 channels, then the amplification of KCa activity and subsequent hyperpolarization. Apamin has been shown to recover CyPPA diminished contractions in murine models\textsuperscript{27}, increase rat myometrial contractility\textsuperscript{18} and have no significant effect on human myometrium\textsuperscript{167}. We theorized that KCa2.3 channel blockade in the presence of augmented internal store release would have a pro-contractile effect due to the decrease in repolarizing effects. Statistical analysis did not support this idea, nor did the trendlines, as apamin with a GPCR or PLC agonist rarely had effects greater than the agent alone. A lack of effect with KCa2.3 blockade is likely due to it not being the main component of repolarization for myocytes. Potassium efflux is required for relaxation but there was many other potassium channels that can continue with potassium efflux if KCa2.3 is inhibited.

Further studies could begin to assess additional relationships between KCa2.3 channels and other channels that have regulatory effects on uterine contractility. Interesting studies have identified the TRPV family as potential modulators of contractions and a relationship between TRPV channels and KCa2 channels in the uterus may yield fascinating findings.

**Conclusions**

Human uterine contractions are different in every patient, not only within gestational groups, but between them as well. Frequencies and amplitudes are not the
same with distributions being more skewed in pregnant tissue. CyPPA relaxes uterine tissue regardless of gestational state. These results were significant across the board with frequency, amplitude, and overall contractility, but individual responses varied in respect to the magnitude of response. CyPPA was also more effective at decreasing contraction amplitude in pregnant tissue. SERCA blockade and internal store depletion did not hamper CyPPA’s abilities to relax contractions but doing so caused waveform changes in a number of samples identifying a potential role of internal stores. CyPPA not only blunts oxytocin's pro-contractile response but can significantly diminish pregnant myometrial contractions in its presence.

Findings from this project have shown many areas of variability with human myometrial tissue, which I perceive to be caused by the differences in cellular architecture noted on immunofluorescence. The expression and distribution patterns of regulating channels are going to lead to functional changes, and with the variety of staining patterns that were visualized amongst and between non-pregnant and pregnant groups, the functional differences aren’t that surprising.

A big question surrounding this study was: are pieces of tissue predictive of the whole organ? Based on the underlying goal of a pregnant uterus to synchronize and coordinate contractions to effectively move the fetus, I do not foresee tissue samples being representative of the whole organ. Currently we do not have methods to safely assess contractile components of an entire uterus therefore we must work with the variability compounded by tissue sample creation and innate differences in cellular architecture amongst patients.
Investigators can work with the variability and further separate gestational states into smaller sub-groups and study those accordingly, because non-pregnant and pregnant human myometrium are not the same. Prescreening uterine tissue for study inclusion could involve assessing if samples functioning within median values, if so it can be grouped with others like it for comparison. Outliers can be identified earlier, and either excluded or grouped with like samples to be further assessed for changes in the contractile apparatus.

Larger studies correlating cellular architecture to functional response can include running parallel data from individual samples, to increase our knowledge on a tissue’s functionality and a corresponding assessment of common channel expression. Accumulation of this information may allow researchers to predict cellular arrangement based on functional characteristics or vice versa. This type of information will help stratify tissue into appropriate sub-groups for researchers to evaluate their scientific questions more effectively.

Evaluation of other channels as possible pharmacological targets for tocolytic agents can prove beneficial to many patients. Based on the variability noted in this study, it is likely a single medication is not going to be the most effective treatment for preterm labor. Multiple agents, each with a distinct and targeted myometrial response, could be used together, to create a more effective tocolytic.
REFERENCES


27. Skarra, D. V., Cornwell, T., Solodushko, V., Brown, A. & Taylor, M. S. CyPPA, a positive modulator of small-conductance Ca\(^{2+}\)-activated K\(^+\) channels, inhibits


49. Thomas, P. Characteristics of membrane progestin receptor alpha (mPRα) and progesterone membrane receptor component 1 (PGMRC1) and their roles in mediating rapid progestin actions. *Front Neuroendocrinol* **29**, 292–312 (2008).


expression in the uterus, pituitary, and forebrain of the female rat.


95. Oliver, D., Klöcker, N., Schuck, J., Baukrowitz, T., Ruppersberg, J. P. & Fakler,


127. Kim, C. J., Weir, B., Macdonald, L. & Zhang, H. Erythrocyte lysate releases Ca$^{2+}$ from IP$_3$-sensitive stores and activates Ca$^{2+}$-dependent K$^+$ channels in rat basilar


133. Zhang, Z., Tang, J., Tikunova, S., Johnson, J. D., Chen, Z., Qin, N., Dietrich, A., Stefani, E., Birnbaumer, L. & Zhu, M. X. Activation of Trp3 by inositol 1,4,5-trisphosphate receptors through displacement of inhibitory calmodulin from a


149. Mendola, P., Nobles, C., Williams, A., Sherman, S., Kanner, J., Seeni, I. & Grantz, K. Air pollution and preterm birth: do air pollution changes over time influence


157. Izumi, H. & Kishikawa, T. Effects of ritodrine, a β2-adrenoceptor agonist, on smooth muscle cells of the myometrium of pregnant rats. *Br J Pharmacol* 76, 463–
471 (1982).


174. Uvnäs-Moberg, K., Ekström-Bergström, A., Berg, M., Buckley, S., Pajalic, Z.,
Hadjigeorgiou, E., Kotłowska, A., Lengler, L., Kielbratowska, B., Leon-Larios, F.,
Magistretti, C. M., Downe, S., Lindström, B. & Dencker, A. Maternal plasma
levels of oxytocin during physiological childbirth-a systematic review with
implications for uterine contractions and central actions of oxytocin. *BMC

175. Padol, A. R., Sukumaran, S. V, Sadam, A., Kesavan, M., Arunvikram, K., Verma,
S. Hypercholesterolemia impairs oxytocin-induced uterine contractility in late
APPENDICES

Appendix A: Supplementary figures

**Figure 40. Secondary antibody only controls.** Samples stained with only the secondary antibody for KCa2.3 channels (A; Alexa Fluor 568) and IP3R channels (B; Alexa Fluor 488) demonstrated no fluorescent signal.
Figure 41. Concentration-response curves for oxytocin, PGF2α, m-3M3FBS, and U73122. Increasing concentrations of oxytocin (A), PGF2α (B), m-3M3FBS (C), and U73122 (D) were added to spontaneously contracting non-pregnant tissue. Nonlinear regression with best first curve was used to demonstrate tissue response as a percentage of maximum AUC.
Appendix B: IRB approval certificate for hysterectomy studies

INSTITUTIONAL REVIEW BOARD
June 7, 2019

Principal Investigator: Mark Taylor, PhD
IRB # and Title: IRB PROTOCOL: 18-173
[1237979-3] Role of KCa2.3 channels in the non-pregnant human uterus
Status: APPROVED
Review Type: Expedited Review
Approval Date: June 4, 2019
Submission Type: Continuing Review
Initial Approval: June 12, 2018
Expiration Date: June 11, 2020
Review Category: Category: 45 CFR 46.110 (9): Continuing review of research, not conducted under an investigational new drug application or investigational device exemption where categories two (2) through eight (8) do not apply but the IRB has determined and documented at a convened meeting that the research involves no greater than minimal risk and no additional risks have been identified

This panel, operating under the authority of the DHHS Office for Human Research and Protection, assurance number FWA 00001602, and IRB Database #00000286, has reviewed the submitted materials for the following:

1. Protection of the rights and the welfare of human subjects involved.
2. The methods used to secure and the appropriateness of informed consent.
3. The risk and potential benefits to the subject.

The regulations require that the investigator not initiate any changes in the research without prior IRB approval, except where necessary to eliminate immediate hazards to the human subjects, and that all problems involving risks and adverse events be reported to the IRB immediately!

Subsequent supporting documents that have been approved will be stamped with an IRB approval and expiration date (if applicable) on every page. Copies of the supporting documents must be utilized with the current IRB approval stamp unless consent has been waived.

Notes:

Expeditied review and approval for the continuation of research granted for one additional year to retain the anniversary of the expiration date of the initial IRB approval; where the research remains ongoing and open to additional enrollment.
Appendix C: IRB approval certificate for cesarean studies
BIOGRAPHICAL SKETCH

Name of Author: Bri Kestler

Graduate and Undergraduate Schools Attended:

University of Portland
Portland, Oregon

Wake Forest School of Medicine
Winston-Salem, North Carolina

University of South Alabama
Mobile, Alabama

Degrees Awarded:

Bachelor of Science in Biochemistry; 2003, Portland, Oregon

Master of Medical Sciences in Physician Assistant Studies; 2010, Winston-Salem, North Carolina

Doctor of Philosophy in Basic Medical Science; Physiology and Cell Biology, 2021, Mobile, Alabama

Awards and Honors:


Cell and Molecular Physiology Section Research Recognition Award
Presented by the American Physiological Society
Experimental Biology Conference 2021

Publications:


