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Role of Potassium Channels in Oocyte Maturation

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ABSTRACT

Ovulation is a critical process for female reproduction. In the majority of mammals, oocyte maturation occurs during ovulation when the oocyte resumes meiosis and advances to metaphase II. In response to the preovulatory gonadotropin surge, cumulus cells produce hyaluronic acid that binds to maturing oocytes. Cumulus expansion occurs when hyaluronic acid gets hydrated, the gaps between cumulus cells expand, and the cells become embedded in a sticky, mucified matrix. Cumulus expansion is one of several necessary processes in preovulatory follicles for ovulation to occur. If either oocyte maturation or cumulus expansion is inhibited, the ovulation rate is drastically decreased. Hence, both of these processes are good targets for novel contraceptive development. Previous studies have demonstrated the presence of a voltage-activated K^+ current with a large conductance in unfertilized eggs. However, the role of potassium channels in oocyte maturation and cumulus expansion is largely unexplored. In this study, the role of K^+ channels in oocyte maturation and cumulus cell expansion has been investigated. Potassium channel inhibitors such as TEAC, valinomycin, and PA-6 were found to reduce cumulus growth in oocytes. To examine the effect of specific potassium transport inhibitors and activators, their ability to inhibit morphological expansion and the expression of expansion genes (*HAS2, PTGS2, PTX3,* and *TNFAIP6*) were assessed. The three potassium channel inhibitors were shown to significantly reduce expansion transcript expression. Our data demonstrate that potassium channels play a crucial role in cumulus expansion and oocyte maturation and that these channels might be a target for the development of new contraceptives.

TABLE OF CONTENTS

LIST OF FIGURES iii

LIST OF TABLES iv

ACKNOWLEDGEMENTS v

Chapter 1 Introduction 1

- Follicular Development 1
- Cumulus cell expansion 2
- Oocyte Maturation 3
- Potassium Channels 4

Chapter 2 6

- Objective Statement 6

Chapter 3 Methods 7

- Animals 7
- Experimental Design 7
- Isolation of Oocytes and Cumulus-Oocyte Complexes 8
- Experiments 8
- Immunostaining of COCs 8
- Spindle Staining 9
- RNA Isolation 10
- RT-PCR 11

Chapter 4 Results 12

- Effect On Cumulus cell expansion in the presence of K+ channel modulators. 12
- Expression of cumulus expansion-related transcripts in Oocytes treated with K+ channel modulators. 14
- Meiotic Stages of Oocytes treated with K+ channel modulators. 15
- Expression of KCNJ2 in oocytes 17

Chapter 5 Discussion 19

- Major Findings 19
- Limitations and Future Questions 21

BIBLIOGRAPHY 23

ACADEMIC VITA 29

LIST OF FIGURES

Figure 1. Expansion potential of oocyte-granulosa cell complexes (COC) from antral follicles in the control medium. 12

Figure 2. Expansion potential of oocyte-granulosa cell complexes (COC) from antral follicles in the presence of 10mM TEAC. 12

Figure 3. Expansion potential of oocyte-granulosa cell complexes (COC) from antral follicles in the control medium and in 5nM, 10nM, 25nM, and 50nM valinomycin. 13

Figure 4. Expansion potential of oocyte-granulosa cell complexes (COC) from antral follicles in the control medium and in 125nM, 250nM, 500nM, 1uM, and 2uM PA-6. 14

Figure 5. Expression of Ptgs2, Ptx3 and Tnfaip6 mRNAs in COCs cultured with control medium, EGF and EFG+PA-6 (10µM)..15

Figure 6. Expression of Ptgs2, Ptx3 and Tnfaip6 mRNAs in COCs cultured with control medium, EGF and EFG+TEAC (10nM)...15

Figure 7. fluorescent images of oocytes cultured with control medium, 5nM, 10nM, and 25nM Valinomycin. Polar body formation is seen in control and 5nM Valinocymin ooctyes. .16

Figure 8. Representative brightfield and fluorescent images of oocytes cultured with (a) PA-6 and (B) TEAC for 15 hours. 16

Figure 9. Immunostaining for KCJN2 inward rectifying channel in GV (A) and MII (B) oocytes. KCNJ2 signal is in green, DNA in blue, and actin in red. 18

LIST OF TABLES

Table 1. The number of oocytes at the germinal vesicle (GV), metaphase I (MI), and metaphase II (MII) stages after 15 hours of in vitro maturation:..17

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Chapter 1

Introduction

Follicular Development

Follicles are the main functional components of the ovary in mammals. The development of ovarian follicles is a complex process that involves multiple stages. The process of follicle development begins with the assembly of primordial follicles and ends with the formation of antral follicles and ovulation. The number of ovarian follicles in females is fixed after birth (Xiong et al., 2019). First, the primordial follicles form from oocytes and pre-follicular cells that were not previously connected. This needs the expression of a factor in the germline alpha (FIGLA), a transcription factor exclusive to germ cells. Next is the activation of primordial follicles into the preantral pool that actively grows. This leads to the development of primary (single cuboidal granulosa cell layer) and secondary (multiple granulosa cell layer) stages of preantral follicles. A primary follicle oocyte enters its extensive development phase, and the follicular cells around it (now known as granulosa cells) change shape to become cuboidal and proliferative. A follicle is referred to as a secondary follicle when the developing oocytes are surrounded by more than one layer of granulosa cells. The primary and secondary follicles are responsive to gonadotrophins, and therefore optimal development of preantral follicles may require these hormones. However, they can still develop in the absence of these hormones (Eppig et al., 2001). Lastly, the change from preantral to the antral follicle is the third important step in the development of large secondary preantral follicles (Diaz et al., 2006).

The change from preantral to antral follicles, a third significant developmental milestone, occurs in large secondary preantral follicles. During this period, the preantral follicle transforms into an antral follicle, which is characterized by the formation of a fluid-filled cavity called the antrum. After the

formation of a fluid-filled antrum, this developmental stage involves the morphological and functional division of preantral granulosa cells into cumulus granulosa and mural granulosa cells. The phenotypes of these two cell types are distinct from those of preantral granulosa cells as well as from one another (Diaz et al., 2006). Cumulus cells, however, seem to be more closely linked to preantral granulosa cells from big secondary follicles since they both express the amino acid transporter SLC38A3 (Eppig et al., 2005), generate anti-Mullerian hormone (AMH) and strongly stain for a surface antigen (OA-1) but not mural granulosa cells (Diaz et al., 2006). Ovulation, along with cumulus cell expansion, is the last significant follicular change.

Cumulus cell expansion

A series of events leading up to ovulation is triggered by the gonadotrophin surge during preovulation. Following the preovulatory surge of Luteinizing hormone (LH), cumulus cells undergo another transformation. This surge starts a chain of events that results in cumulus expansion, which is a distinguishing property of cumulus cells (Diaz et al., 2006). Cumulus expansion, or mucification, is one of these processes that stands out the most. Hyaluronic acid is a non-sulfated glycosaminoglycan that binds to cumulus cells and enlarges the gaps between the cells (Eppig et al., 2001). This embeds the cumulus cells in a mucinous matrix. Hyaluronic acid is produced by cumulus cells when gonadotrophins are present. This mechanism is essential for ovulation because blocking hyaluronic acid production or connecting cumulus cells *in vivo* significantly decreases ovulation rates (Chen et al., 1993). Cumulus cell differentiation is evidenced by the production of cumulus marker transcripts before the LH surge and the capacity to undergo cumulus expansion after the LH surge.

Growth of the cumulus oophorous is caused by a preovulatory surge of LH *in vivo* or by Epidermal Growth Factor (EGF) or Follicle Stimulating Hormone (FSH) treatment *in vitro*

and includes MAPK3/1-dependent upregulation of *Has2, Ptgs2, Ptx3, and Tnfaip6* mRNAs, as well as cumulus cell synthesis of a mucified matrix (Diaz et al., 2006, Su et al., 2003). Since null mutations in *Ptgs2, Ptx3, or Tnfaip6* or pharmacological suppression of these mRNAs expression all result in insufficient or faulty cumulus expansion, each of these transcripts is required for cumulus expansion (Chen et al., 1993, Fulop et al., 2003, Ochsner et al., 2003a, Ochsner et al., 2003b, Varani et al., 2002). Cumulus-expansion enabling factors released by fully-grown oocytes are also necessary for growth, highlighting the complicated control of cumulus expansion (Buccione et al., 1990, Diaz et al., 2006, Vanderhyden et al., 1990).

Both oocyte and cumulus cell activities are controlled by paracrine signaling in the cumulus-oocyte complex. Oocytes define the cumulus cell phenotype by promoting gene expression (Diaz et al. 2007), cholesterol synthesis (Su et al. 2008), proliferation (Gilchrist et al. 2006), glycolysis (Sugiura et al. 2005), and survival (Hussein et al. 2005) while inhibiting luteinization (Diaz et al. 2007). Oocytes promote the expression of the epidermal growth factor (EGF) receptor, which allows MAKP3/1 signaling in cumulus cells (Su et al., 2010). Cumulus cells, in turn, feed nutrients to the oocyte (Eppig et al. 2005, Sugiura et al. 2007, Su et al. 2008), cause transcriptional silencing (Eppig 2001), and prevent meiosis from resuming before ovulation (Zhang et al. 2010). As a result, oocyte growth and fertility require mutual and multiple interactions between oocyte and cumulus cells (R S Lisle, 2013).

Oocyte Maturation

Oocyte maturation is the process through which an immature oocyte develops into a mature ovum and occurs during ovulation. It is accompanied by the end of meiosis I and the beginning of meiosis II. Meiosis I begins in the fetal stage and continues through the prophase until the ovulatory phase. At this stage, the oocyte's nucleus, known as the germinal vesicle (GV), is still intact, and the oocyte is known as a GV oocyte. The chromosomes have replicated, and crossing over has occurred, but the oocytes remain

arrested at prophase I of meiosis 1. Oocyte arrest is maintained by cyclic nucleotides (cAMP and cGMP). High intracellular cAMP levels are maintained by oocyte-mediated synthesis as well as cAMP influx generated by nearby Cumulus cells (Turathum et al., 2021). When the ovulatory phase occurs, the LH surge triggers the resumption of meiosis. In response to the LH surge, cumulus cells produce EGFs such as amphiregulin (AREG), epiregulin (EREG), BTC, and PTGE2 during the LH surge, which blocks the formation of cGMP and cAMP. This is followed by a decrease in cAMP in the oocyte and the restart of meiotic progression via protein kinase A (PKA) dephosphorylation and activation of the mitotic promoting factor (MPF), as well as meiotic maturation, nuclear envelope breakdown (Matzuk et al., 2002; Richard, 2005)]. The breaking of the GV is the first observable event that the resumption of meiosis has begun. The oocyte then divides into a secondary oocyte and a polar body. Meiosis II begins immediately after (Chen et al., 2013). Until fertilization, the oocyte remains in the Meiosis II metaphase (MII). The MII oocyte is a fully matured oocyte that is ready for fertilization (Mehlmann, 2005; Edson et al., 2009).

Besides paracrine signaling, changes in the electrical properties of the plasma membrane and the function and distribution of ion channels are also related to oocyte maturation. Variations in the pattern of expression, distribution, and function of ion channels and transporters during oocyte maturation are, therefore, crucial for reproductive success. Ion channels and transporters are crucial to membrane potential regulation.

Potassium Channels

The potassium (K^+) channel family is the biggest of all ion channels. A crucial characteristic of all K^+ channels is their capacity to selectively permit penetration of K^+ through the membrane at rates close to the diffusion limit. In other words, they accurately distinguish between K^+ and other monovalent cations and anions, allowing K^+ to move into and out of cells. Based on the foundation of K^+ selectivity,

K^+ channels have evolved various gating mechanisms (i.e., opening and closing) and functions in several cell types.

Based on the criteria of the International Union of Basic and Clinical Pharmacology (IUPHAR; Shen et al.2009), the mammalian K^+ channels are classified into four superfamilies: voltage-gated K^+ (K_v) channels, Ca^{2+}-activated K^+ (K_{Ca}) channels, inwardly rectifying K^+ (K_{ir}) channels, and two-pore domain K^+ (K_{2P}) channels. These K^+ channels serve primarily to maintain resting membrane potential and K^+ homeostasis. Mammalian cells govern a number of biological activities through these fundamental tasks, including the regulation of neuronal firing rate and heart rate, muscular contraction, hormone secretion, cell proliferation, and apoptosis (Kim 2005, Lang et al. 2005, Shen et al. 2009, Enkvetchakul 2010).

A family of inwardly rectifying potassium (Kir) channels aids in the maintenance of K+ levels in cells. Kir channels are made up of a tetramer of Kir subunits, each of which has two transmembrane domains and is encoded by the KCNJ gene. At the monomer interface, the formed Kir channel provides an ion selectivity filter for K+, allowing for K+ passage. Kir channels are located in a variety of cell types and control K+ homeostasis throughout the organism, regulating muscle, neuron, and immunological function (Hager et al., 2022). Kir2.1 (KCNJ2), Kir2.2 (KCNJ12), Kir2.3 (KCNJ4), Kir2.4 (KCNJ14), and Kir2.6 (KCNJ18) are all members of the Kir2 subfamily. Many studies have revealed that these K+ channels may govern a vast array of physiological processes in mammalian cells (Buckler 2010, Enyedi & Czirjak 2010); however, the precise role of these channels in oocyte maturation and cumulus expansion remains unknown (Hur et al., 2012).

Chapter 2

Objective Statement

This project aimed to determine the effects of blocking K^+ channels on the process of cumulus cell expansion and oocyte maturation in mice. The results show that both processes require K^+ channel activity which represents a novel finding in the field of reproductive biology.

Chapter 3

Methods

Animals

Outbred CD-1 or B6SJLF1 female mice (Mus musculus) were produced and raised in the research colony of the investigators at Pennsylvania State University and were used for all experiments. Mice ovaries were taken on day 22, 48 hours after an intraperitoneal injection of 5 IU eCG (National Hormone and Peptide Program, NIDDK). eCG was used to stimulate follicular development and ovulation in mice. The mice were maintained in compliance with the Guide for the Care and Use of Laboratory Animals (Institute for Learning and Animal Research) (Diaz et al., 2006).

Experimental Design

In this study, the independent variables were the K+ channel inhibitors such as TEAC, valinomycin, and PA-6. When the COCs were treated with these inhibitors, they were immunostained to be observed for potential cumulus cell expansion. Besides this, the expression of expansion genes *ptgs2, ptx3, and tnfaip6* were assessed through RNA isolation. The genes *ptgs2, ptx3, and tnfaip6* are expressed in normal cumulus cell expansion, therefore, inhibition of these genes would confirm inhibition of cumulus cell expansion. Since cumulus cell expansion plays an essential role in resuming meiosis, spindle staining was done to analyze the fluorescent images of the COCs and see if there were any changes in the spindle formation in the treated COCs.

Isolation of Oocytes and Cumulus-Oocyte Complexes

Cumulus–oocyte complexes (COCs) containing GV-stage oocytes were collected from the mice by first isolating their ovaries and then by puncturing the ovaries gently using a syringe and needle. The cells were then separated and picked by gently pipetting the COCs. For some experiments, the COCs were denuded by pulling the complexes into and out of pipettes and shearing against the bottom of a culture dish until the complexes were separated.

Experiments

Tests of the expansion of COCs and oocytes were performed in 25-µl drops under the oil of bicarbonate-buffered MEM-α supplemented with Earles salts, supplemented with 75 mg/l penicillin G, 50 mg/l streptomycin sulfate, 0.23 mM pyruvate. Untreated COC were used as controls and were compared to COCs treated with EGF alone (10 ng/ml) to induce cumulus expansion or EGF plus one of the following k channel modulators: valinomycin (25n M), PA-6 (10 µm), or TEAC (10 mM). All experiments were performed at least 3 times.

Immunostaining of COCs

The COCs and oocytes were fixed in 4% paraformaldehyde in PBS for 30 minutes. It was then permeabilized for 15 minutes with PBST in addition to 0.2% Triton X-100. The samples were then washed in PBS with Tween 20 containing 1% BSA. The samples were then blocked in a goat serum blocking Buffer containing PBS, 2% goat serum, 1% BSA, 0.1% Triton X-100, and 0.05% Tween 20 for 1 hour at 37°C. The COCs and the oocytes were incubated for 1 hour with primary antibody Anti-Lamp1 which was diluted 1:500 in PBS at 37°C or overnight at 4°C. Next, the samples were washed three times in PBST containing 1% BSA. They were then incubated for 1 hour at 37°C with the secondary antibody

Goat-anti Rabbit-488, which was diluted 1:1000. The samples were again washed three times in PBST containing 1% BSA. The cells were then mounted in 4',6-diamidino-2-phenylindole (DAPI) anti-fade gold (Invitrogen, Carlsbad, CA) on glass slides with etched rings to prevent them from being ruptured by the coverslip. The edges of the slides were then sealed with nail polish.

Spindle Staining

The oocytes were fixed in a microtubule-stabilizing Buffer containing 2.5 mM EGTA, 5 mM MgCh, 0.1 mM PIPES, 2% paraformaldehyde, and 2.5 mM Triton X-100 for 30 minutes at 37°C. After fixing, oocytes were washed three times in PBS with Tween 20 containing 1% BSA. The samples were then blocked in goat serum blocking Buffer containing PBS, 2% goat serum, 1% BSA, 0.1% Triton X-100, and 0.05% Tween 20 for 50 min at 37°C. This was followed by incubation with anti-a-tubulin (1:200; Sigma Chemical Co., St. Louis, MO) and anti-v-tubulin (1:200; Sigma) for 1 hour at 37°C. The samples were again washed three times in PBS with Tween 20 containing 1% BSA. The oocytes were incubated with goat anti-mouse IgG Alexa Fluor 488 (1:1000; Molecular Probes, Eugene, OR) and phalloidin-tetraethylrhodamine isothiocyanate (TRITC) (10 g/ml; Sigma) for 30 min at 37°C followed by washing (three times) with PBST. Oocytes were then mounted in 4',6-diamidino-2-phenylindole (DAPI) anti-fade gold (Invitrogen, Carlsbad, CA) on glass slides with etched rings to prevent oocytes from being ruptured by the coverslip. The slides were imaged with an epifluorescent microscope (AxioScope 2 Plus; Leica, Bannockburn, IL) and a DP20 Olympus digital color camera and DP software or by confocal microscopy using an Olympus Fluoview 1000 confocal microscope.

RNA Isolation

To assess the *Ptx3, Ptgs2,* and *Tnfaip6* mRNA levels, RNA was isolated from COCs using the RNA isolation kit (RNAspin mini total RNA isolation kit) according to the manufacturer's protocol. To begin the RNA isolation process, the first homogenization and lysis of the cells were done. 350µl lysis solution and 3.5 µl β-mercaptoethanol were added to the frozen cells. A rotor-stator was used to disrupt the cells. To reduce the viscosity and clear the lysate, filtration was done through an RNAspin Mini Filter. The lysate was added to the RNAspin Mini Filter and centrifuged at 11000 × g for 1 minute. To adjust the RNA binding condition, 350 µl of 70% ethanol was added and vortexed for 5 seconds each. The lysate was then pipetted into the RNAspin Mini Column and centrifuged for 30 seconds at 8000×g. This was done for RNA binding. Next, 350 µl of Desalting Buffer was added and centrifuged for 1 minute at 11000×g. Desalting is important as it makes the DNase digestion much more effective. For DNA digestion, DNase 1 reaction mixture was prepared by adding 10 µl reconstituted DNase 1 to 90 µl DNase Reaction Buffer for each isolation. 95 µl of the reaction mixture was added to each column and was allowed to incubate at room temperature for 15 minutes. To inactivate the DNase, the RNAspin Mini Column was then washed using 200 µl of Wash Buffer 1 and centrifuged for 1 minute at 11000×g. This was followed by a second wash using 600 µl of Wash Buffer 2 and centrifuged for 1 minute at 11000×g. For the last wash, 250 µl of Wash Buffer 2 and centrifuged for 2 minutes at 11000×g. This also completely dried the membrane. To elute the RNA, 25 µl of RNase-free H2O was added and centrifuged for 1 minute at 11000×g. Eluted RNA was placed immediately on ice to prevent potential degradation. 22.5 µl of the isolated RNA was mixed with 1.5 µl of reverse transcriptase and 5 µl of 5x Buffer. It was frozen in thin-walled PCR tubes until required for RT-PCR (Winston 2006).

RT-PCR

Using an ABI 7500 real-time PCR equipment, *Ptx3, Ptgs2,* and *Tnfaip6* mRNA levels were quantified. The sequences of PCR primers were created with Primer Express software and have been validated previously (Diaz et al., 2006). Amplification processes were carried out in a 10 µl reaction volume comprising 1µl of cDNA, 5µl of 2x PCR mix, 2µl of RNase-free water, and 1µl each of forward and reverse gene-specific primers. The conditions for cycle amplification were as follows: 10 min at 95°C, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 min. PCR products were sequenced, the dissociation curve examined, and gel electrophoresed immediately after amplification to determine the specificity of the reaction. Expression of Rpl19 mRNA was utilized to normalize specific expression of, *Ptgs2, Tnfaip6,* and *Ptx3* mRNA based on the formula 2-(Ct gene of interest- Ct Rpl19), where Ct is the cycle number at which the fluorescence intensity of each sample exceeds a predetermined threshold level over the background. All experiments were replicated three times (Diaz et al., 2006).

Chapter 4

Results

Effect On Cumulus cell expansion in the presence of K+ channel modulators.

In the presence of TEAC, PA-6, and valinomycin, the cumulus cell expansion in the COC was inhibited at different concentrations. After 15h incubation of COCs in a control expansion medium (10 ng/ml EGF + 5% Serum) and expansion medium containing 10mM TEAC, the control set of COCs exhibited normal cumulus cell expansion (Fig.1), whereas the oocytes in the presence of TEAC exhibited inhibited cumulus cell expansion (Fig.2). The inhibition can be seen in treated COCs (Fig.2) as the cells surrounding the COCs are much less and compact when compared to the COCs in control medium (Fig. 1).

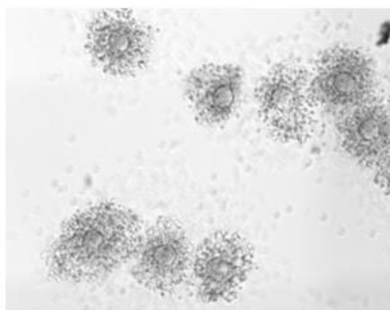

Figure 1. Expansion potential of oocyte-granulosa cell complexes (COC) from antral follicles in the control medium.

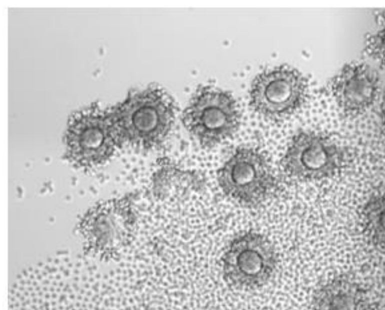

Figure 2. Expansion potential of oocyte-granulosa cell complexes (COC) from antral follicles in the presence of 10mM TEAC.

Similarly, COCs were incubated for 15 hours in a control expansion medium and in the presence of valinomycin at 5nM, 10nM, 25nM, and 50nM concentrations. The results indicated that the expansion of COCs treated with 5nM valinomycin did not differ from the expansion of control cumulus cells.

However, at 10nM, 25nM, and 50nM, cumulus cell growth in the COCs was clearly inhibited (Fig.3). The inhibition can be seen as the cells surrounding the oocyte is less expanded and clumped together.

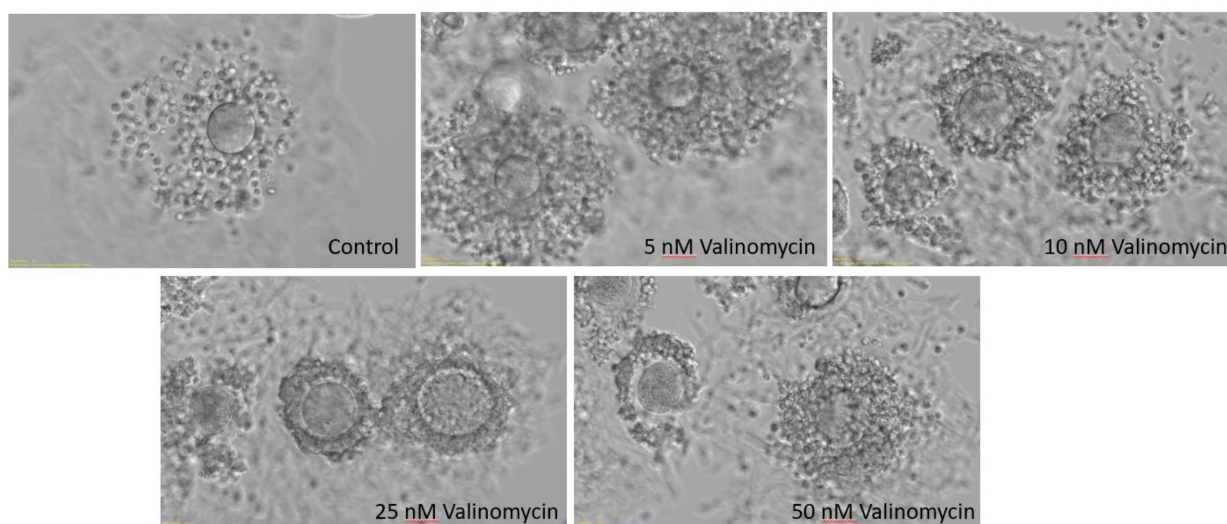

Figure 3. Expansion potential of oocyte-granulosa cell complexes (COC) from antral follicles in the control medium and in 5nM, 10nM, 25nM, and 50nM valinomycin.

When the COCs were treated with different concentrations of PA-6 and incubated for 15hr, different effects on cumulus cell expansion were observed. When compared to the control COCs, cumulus cell expansion in COCs treated with 125 nM did not show any difference. However, at 250 nM and 500 nM concentrations of PA-6, inhibition of cumulus cell expansion was seen. At 1µM and 2µM concentrations of PA-6, expansion was inhibited, with the cumulus cells becoming clumped together and appearing darker in color (Fig. 4).

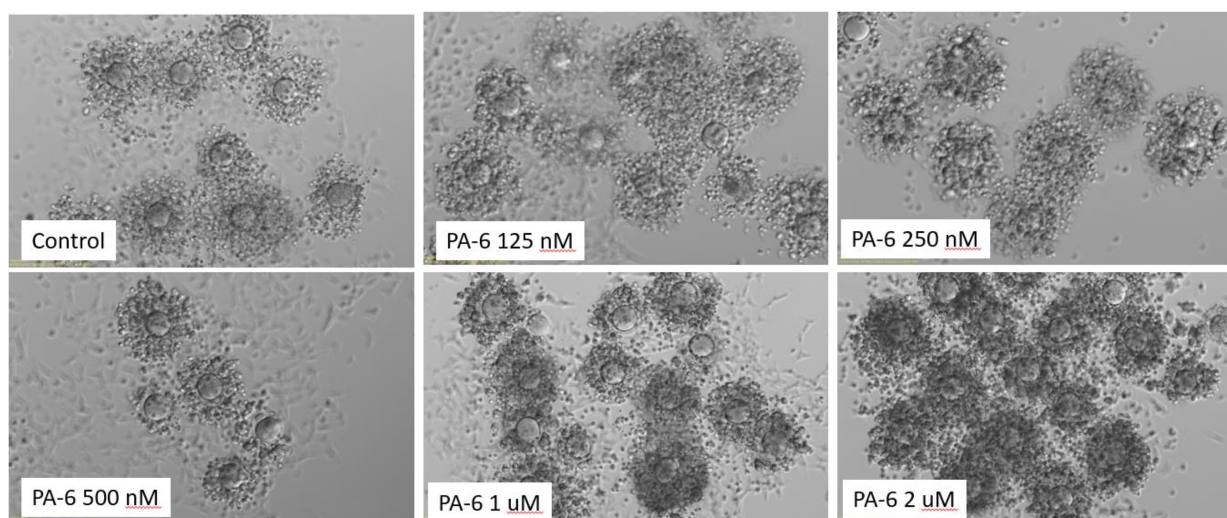

Figure 4. Expansion potential of oocyte-granulosa cell complexes (COC) from antral follicles in the control medium and in 125nM, 250nM, 500nM, 1uM, and 2uM PA-6.

Expression of cumulus expansion-related transcripts in Oocytes treated with K+ channel modulators.

Previous research has demonstrated that *Ptgs2, Ptx3, and Tnfaip6* mRNA levels are increased during cumulus expansion (Diaz et al., 2007). So, to assess the effect of the K^+ channel modulator effect, the expression of these genes was investigated in the treated COCs. The expansion of cumulus cells is induced by the preovulatory surge of LH *in vivo* or by EGF treatment *in vitro* (Diaz, 2007). Therefore, the levels of *Ptgs2, Ptx3, and Tnfaip6* mRNA were assessed in fresh COCs (before expansion), COCs cultures with EGF, and COCs cultured EGF along with either PA-6 or TEAC.

It was found that for the PA-6 modulator, *Ptgs2* mRNA expression was significantly inhibited for the EGF+PA-6 COC group when compared to the EGF-treated COCs (Fig.5). Unexpectedly, PA-6 increased *Ptx3* mRNA expression EGF+PA-6 COC group when compared to the EGF treated COCs (Fig.5). However, *Tnfaip6* mRNA expression was significantly inhibited for EGF+PA-6 COC group when compared to the EGF treated COCs (Fig.5). For the TEAC K+ channel modulator, expression of all three *Ptgs2, Ptx3, and Tnfaip6* mRNA levels was significantly inhibited for EGF+TEAC COC group

when compared to the EGF treated COCs (Fig.6). Expansion transcripts were analyzed by ANOVA followed by Tukey's post-hoc test.

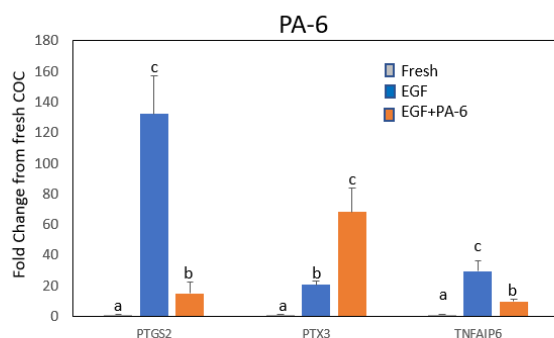

Figure 5. Expression of Ptgs2, Ptx3 and Tnfaip6 mRNAs in COCs cultured with control medium, EGF and EFG+PA-6 (10µM)

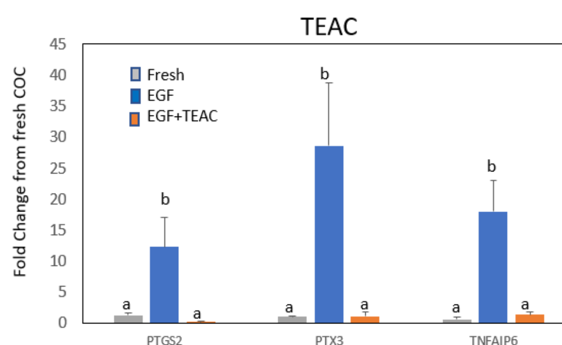

Figure 6. Expression of Ptgs2, Ptx3 and Tnfaip6 mRNAs in COCs cultured with control medium, EGF and EFG+TEAC (10nM)

Meiotic Stages of Oocytes treated with K^+ channel modulators.

The oocytes were cultured in a control medium, Valinomycin, TEAC, and PA-6, for 15hr and then the spindle staining process was performed on them. This was done to analyze at which stage of meiosis the oocyte was. As previously mentioned, the cumulus cells play a major role in resuming meiosis when the oocyte then divides into a secondary oocyte and a polar body. The bright field and fluorescent images of the oocytes were used to assess the meiotic stage of the oocytes.

The oocytes were treated in a control medium or in the presence of valinomycin at 5nM, 10nM, 25nM, and 50nM concentrations. When fluorescent images of these oocytes were observed, it was found that the control oocyte had resulted in the formation of a polar body, as expected. Also, the oocytes treated with 5nM valinomycin showed a formation of a polar body which is consistent with the cumulus expansion results. Interestingly, the oocytes treated in 10nM and 25nM concentrations of valinomycin did not exhibit the formation of any polar body (Fig. 7). Similarly, bright field and fluorescent images of the oocytes treated with PA-6 (10µM) and TEAC (10mM) were observed. It was found that both these K^+ channel inhibitors inhibited the formation of a polar body after 15 hours of incubation (Fig 8).

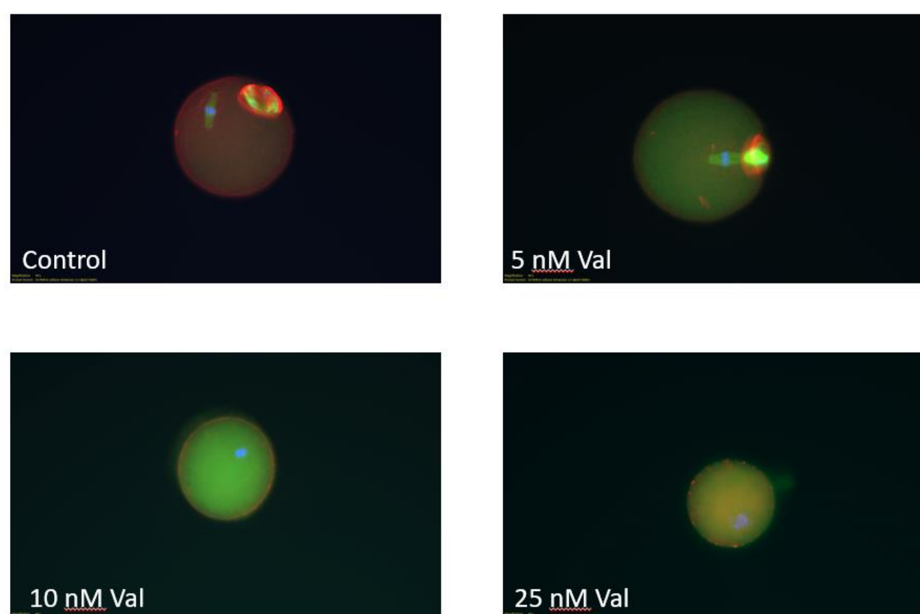

Figure 7. fluorescent images of oocytes cultured with control medium, 5nM, 10nM, and 25nM Valinomycin. Polar body formation is seen in control and 5nM Valinocymin ooctyes.

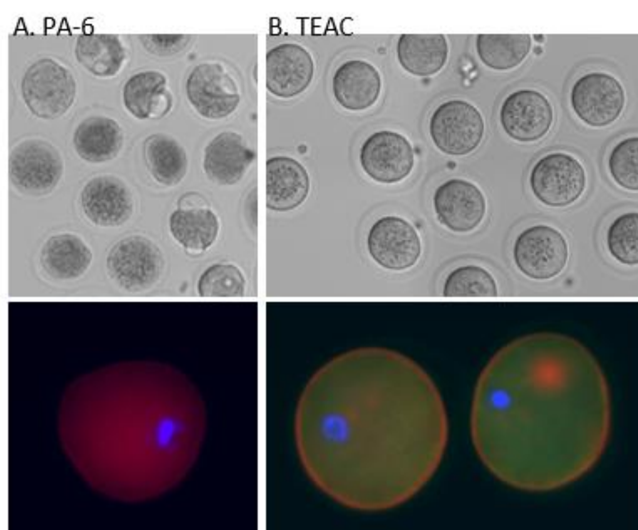

Figure 8. Representative brightfield and fluorescent images of oocytes cultured with (a) PA-6 and (B) TEAC for 15 hours.

The stages of the oocytes were recorded and are shown below (Table 1). It was seen that after 15 hours of *in vitro* maturation, 43% of the oocytes in the positive control medium were at the Metaphase II stage, whereas no oocytes cultured in either PA-6, Valinomycin, or TEAC were present at the Metaphase II stage.

Table 1. The number of oocytes at the germinal vesicle (GV), metaphase I (MI), and metaphase II (MII) stages after 15 hours of *in vitro* maturation. Analyzed by Chi-squared test:

	GV	MI	MII	Proportion MII	P-value (vs. PC)
Positive control	1	110	83	43%	.
PA-6 10 μM	1	206	0	0	< 0.0001
Valinomycin 25 nM	5	120	0	0	< 0.0001
TEAC 4 mM	4	100	0	0	< 0.0001

Expression of KCNJ2 in oocytes

As previously mentioned, KCNJ2 is one member of the kir2.x family that is blocked by PA-6. To investigate if KCNJ2 was present in the oocyte and where it was expressed, the oocytes were immunostained for KCNJ2 inward rectifying channel in both GV and MII oocytes. When the fluorescent images were observed, it showed an unexpected expression of KCJN2 in the perinuclear area in GV oocytes and on the spindle in MII eggs (Fig.9).

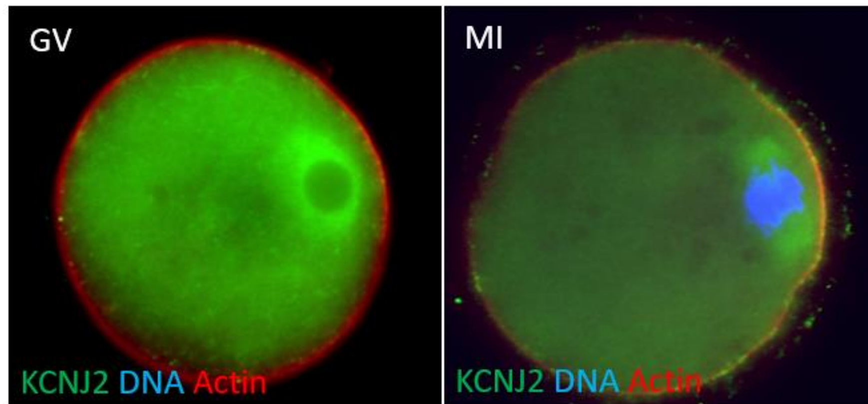

Figure 9. Immunostaining for KCJN2 inward rectifying channel in GV (A) and MII (B) oocytes. KCNJ2 signal is in green, DNA in blue, and actin in red.

Chapter 5

Discussion

Major Findings

The production of cumulus marker transcripts before the LH surge and the capacity to undergo cumulus expansion following the LH surge are indicators of cumulus cell expansion. If cumulus cell growth is inhibited *in vivo*, ovulation rates are significantly reduced (Chen et al., 1993). In addition, cumulus cells play a significant role in oocyte maturation when the oocyte resumes meiosis and divides into a secondary oocyte and a polar body. Recent research has shown that unfertilized eggs have a voltage-activated K^+ channel with a high conductance (Carvacho et al., 2018), but the involvement of K^+ channels in either cumulus expansion or oocyte maturation is not known. In this study, the modulators of potassium channels were utilized in an *in vitro* model to examine the role of potassium channels in cumulus cell expansion and oocyte maturation. The effect of potassium channel inhibitors such as TEAC, valinomycin, and PA-6 was examined. This was accomplished by assessing their ability to inhibit morphological expansion and the expression of expansion genes (*Ptgs2, Ptx3, and Tnfaip6*), as well as by examining fluorescent images to determine whether COCs treated with these inhibitors could resume meiosis. Because EGF stimulates cumulus cell expansion *in vitro* (Diaz et al., 2006), it was used to examine the ability of cultured complexes to undergo cumulus expansion.

TEAC is a non-specific k-channel inhibitor. When the oocytes were treated with 10mM TEAC, the expansion of cumulus cells was clearly inhibited. This was validated when

EGF+TEAC suppressed the expression of *Ptgs2, Ptx3, and Tnfaip6* mRNA in EGF+ TEAC-treated COCs. Previous research has demonstrated that cumulus cell growth is essential for the resumption of meiosis and the development of the polar body. The absence of a polar body in the fluorescent pictures of oocytes treated with 10mM TEAC was consistent with the prior two findings. This demonstrates that the TEAC-inhibited K^+ channels are essential for cumulus expansion and oocyte maturation.

To investigate whether overloading cells with potassium interfered with cumulus expansion, we treated COC with the K^+ ionophore valinomycin. Cumulus expansion of COCs treated with 5nM valinomycin was not suppressed, indicating that this concentration was insufficient to block the targeted K^+ channels. However, at higher concentrations of valinomycin of 10nM, 25nM, and 50nM, the expansion of cumulus cells in the COCs was clearly suppressed. The absence of polar body development in fluorescent pictures of oocytes treated with 10nM, 25nM, and 50nM concentrations of valinomycin confirmed that valinomycin also inhibited oocyte maturation. In contrast, oocytes treated with 5nM valinomycin displayed the predicted development of a polar body. This demonstrated that valinomycin at concentrations higher than 5nM effectively inhibited oocyte maturation. How both loading cells with potassium using valinomycin and blocking k-channel activity with TEAC lead to suppressed cumulus expansion and oocyte activation is not precisely known, and if too much or too little interferes with membrane polarity. Another possibility is that TEAC is acting at the cell surface only, while valinomycin can enter the cells and could affect intracellular k-channels.

Other work in the lab identified PA-6 as a specific k-channel inhibitor in granulosa cells. PA-6 blocks the inward rectifying k channels of the Kir 2.X family, which has 4 members (KCNJ2, KCNJ4, KCNJ12, and KCNJ14). Addressing the PA-6 potassium channel inhibitors,

the expansion of cumulus cells in COCs treated with 125 nM PA-6 was not inhibited. At 250 nM, 500 nM, 1 M, and 2 M doses of PA-6, the expansion of cumulus cells was inhibited. The inhibition of the expression of Ptgs2, Ptx3, and Tnfaip6 mRNA in oocytes treated with EGF+PA-6 yielded variable results. PA-6 actually increased the expression of Ptx3 mRNA but significantly suppressed the expression of Ptgs2 and Tnfaip6. The expression of all three expansion genes was anticipated to be inhibited. Due to the lack of prior research on the function of potassium channels in oocytes, the cause of this result is not entirely understood. However, the fluorescent pictures of oocytes treated with PA-6 did not demonstrate the formation of a polar body, as predicted. Localization of KCNJ2 might yield some clues. In GV oocytes, KCNJ2 localized to the perinuclear area, which included the ER and nuclear envelope. This might mean that transport of k ions across this membrane is important for GVBD and oocyte maturation. In MII eggs KCNJ2 localized to the spindle, which was unexpected and could signify the presence of this channel on vesicular components of the spindle, such as lysosomes. These could be important for chromosome separations or condensation.

Limitations and Future Questions

This study provides adequate evidence to suggest that potassium channels are essential for cumulus cell expansion and oocyte maturation. Blocking potassium channels will inhibit the expansion of cumulus cells and the maturation of oocytes, including chromosome condensation and spindle formation. However, regulation of the expression of only three expansion genes, Ptgs2, Ptx3, and Tnfaip6, was investigated in this study. Has2 is an additional key expression gene that requires investigation. The investigation of how potassium channel inhibitors influence

the expression of Has2 will provide additional evidence for the role of potassium channels in the regulation of cumulus cell growth. Knowing how TEAC, PA-6, and valinomycin interact with signaling pathways during cumulus growth could explain the unexpected rise in Ptx3 expression caused by PA-6. The influence of FSH and LH levels on potassium channels could bring additional insight into this field of study. The action of intracellular k-channels for the Kir2.x family warrants further research as the regulation of intracellular k channels has not been thoroughly investigated. Finally, if these compounds were to be used as potential contraceptives, it would be important to know the potential side effects and also test for their toxicity levels.

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