GONADOTROPIN-INDUCED STEROIDOGENESIS AND DOWNSTREAM SIGNALS LEADING TO OOCYTE MATURATION

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DEDICATION

I would like to dedicate this dissertation to my parents and sister who have supported and loved me throughout this process.

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by

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

August, 2008

Acknowledgements

I could not have completed this research without the help of my mentor Stephen Hammes who allowed me to become a better scientist and also all the members of the lab during the time I was there who became a second family to me.

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The University of Texas Southwestern Medical Center at Dallas, 2008

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The Hammes laboratory is interested in understanding the process of steroid-mediated oocyte maturation. This includes examining both steroid production and steroid signaling. In these studies, gonadotropin-induced steroid production was examined in the gonads using mouse models, as well as steroid-induced oocyte maturation in frog models. cAMP signaling is known to be important for steroid production, but further downstream pathways were not well characterized. These studies illuminate other downstream signaling pathways triggered by luteinizing hormone (LH) that regulate steroid production in the testes using Leydig cells, which are the primary steroidogenic cells in the testes.

A novel downstream pathway was found involving epidermal growth factor receptor (EGFR) transactivation, downstream mitogen-activated protein kinase (MAPK) and steroidogenic acute regulatory protein (StAR) activation that was essential for short, but not long-term LH-induced steroidogenesis in MLTC-1 and primary mouse leydig cells. Despite this discrepancy *in vitro*, EGFR signaling was required *in vivo* for testicular testosterone production. To study the effects of steroids on oocyte maturation, the *Xenopus laevis* frog model was used**.** It has been shown that G-beta gamma, as well as other signals, keep the oocyte in meiotic arrest. Steroids block this constitutive signal, leading to oocyte maturation. To directly measure rapid changes in G-beta gamma signaling in oocytes, G-beta gamma sensitive-inward rectifying potassium channel currents (GIRKS) were exogenously expressed in *Xenopus* oocytes. Adding testosterone, the physiologic mediator of oocyte maturation in *Xenopus*, decreased the G-beta gamma mediated signal. This happened rapidly supporting the well known idea that maturation is a transcription-independent process. It was also seen that the classical androgen receptor (AR) was being used for this process. When the AR was knocked down, testosterone could only decrease GIRK signal at higher concentrations. This showed that testosterone is working, at least partially, through the AR. These studies may help elucidate novel targets for polycystic ovary syndrome (PCOS), which is characterized by excess androgen due to improper steroid production.

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PUBLICATIONS PRESENTED IN THIS THESIS

Evaul K, Hammes SR 2008 Cross Talk Between G Protein-Coupled and Epidermal Growth Factor Receptors Regulates Gonadotropin-Mediated Steroidogenesis in Leydig Cells. The Journal of biological chemistry

Evaul K, Jamnongjit M, Bhagavath B, Hammes SR 2007 Testosterone and Progesterone Rapidly Attenuate Plasma Membrabe Gβγ-Mediated Signaling in *Xenopus laevis* Oocytes by Signaling through Classical Steroid Receptors, Mol Endocrinology 21: 186-196

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LIST OF DEFINITIONS

EGFR – Epidermal Growth Factor Receptor

MMP – Matrix Metalloproteinases

GnRH – Gonadotropin-Releasing Hormone

LH – Luteinizing Hormone

FSH – Follicle Stimulating Hormone

StAR – Steroidogenic Acute Regulatory Protein

PKA – Protein Kinase A

MAPK – Mitogen Activated Protein Kinase

PCOS – Polycystic Ovary Syndrome

AR – Androgen Receptor

PR – Progesterone Receptor

MNAR – Modulator of Nongenomic Action of Estrogen Receptor

hCG – Human Chorionic Gonadotropin

GIRK – G-Protein Regulated Inwardly Rectifying Potassium Channel

M2R – Muscarinic Receptor

Chapter 1

General Introduction

1.1 Epidermal growth factor receptor signaling

Epidermal growth factor receptors (EGFRs) are protein tyrosine-kinase receptors (1). The EGFR family includes: the EGFR, ERBB2, ERBB3 and ERBB4. The members of the EGFR family all contain a cytoplasmic tyrosine kinase domain, a single transmembrane domain, and an extracellular domain for ligand binding and receptor dimerization. ERBB3 has defective kinase activity whereas ERBB2 has no ligand so their function is to help modulate or intensify EGFR or ERBB4 signal. For the other three receptors, there are seven possible ligands: amphireulin (AREG), betacellulin (BTC), heparin-binding EGF like growth factor (HBEGF), transforming growth factor alpha (TGFA), epiregulin (EREG), epigen (EPGN) and EGF. These ligands start off in a membrane bound form and must be cleaved in order to be activated (2). Tyrosine-kinase receptors have been seen to be important for many different functions in mammalian cells such as regulating growth, survival, proliferation, and differentiation (2, 3). Early signals begin with ligand induced EGFR activation and subsequent binding and activation of growth factor receptor binding protein 2 (Grb2) and phosphorylation of Src homology and collagen domain protein (Shc), and Phospholipase C-γ (PLCγ) which leads to many downstream pathways (4). Important downstream signals include mitogen-activated protein kinase (MAPK) signaling, G-protein coupled signaling, phosphatidylinositol phosphate (PIP) signaling and intracellular calcium signaling (2). These downstream signals depend on amplitude, timing and duration of activation of the EGFR (4). The EGFR becomes either a homo-dimer or hetero-dimer with other members of the receptor family after ligand binding and is either autophosphorylated or Shc phosphorylated (5). Later EGFR becomes a clathrin-coated oligomer in order to be internalized for lysosomal degradation. Grb2 has been shown to have a role in regulating the initial steps of

EGFR internalization (6). After internalization, the EGFR is ubiquitinated in order to be targeted for degradation. There is some evidence that the EGFR can still signal once in endosomes so there is a possibility of even further signaling prior to degradation (5). There are several examples of EGFR genetically mutated rodents that link EGFR function with reproduction. Puberty is delayed when the EGFR is inhibited pharmacologically in rats or in mice that have a defective EGFR. Hypothalamic astrocytes express increased levels of ERBB2 and ERBB4 during puberty with an even greater increase before the preovulatory surge of gonadotropins. Also, disruption of the EGFR in astroglia caused by knockin of a mutant receptor in mice caused decreased LH secretion and irregular estrous cycles (2). EGF has been seen to activate reproductive behavior independent of sex steroids in female rodents (7). EGFR signaling is also important in gonads for oocyte maturation (8).

1.2 Matrix metalloproteinases

Matrix metalloproteinases (MMPs), sometimes called matrixins, are part of the bigger family of zinc-dependent endopeptidases called metzincins. MMPs have a prodomain, catalytic zinc domain, hinge region and a c-terminal hemopexin domain. They can degrade extracellular matrix proteins to allow for many different functions including reproduction, embryogenesis, wound healing, and angiogenesis (9, 10). MMP activation causes cleavage of ectodomains which allows for step-wise signaling. In general, MMPs cleave a peptide bond before a residue with a hydrophobic side chain such as Leu, Ile, Met, Phe or Tyr (10). There are several different types of MMPs. The most common are Collagenases (MMP1/8/13) which cleave interstitial collagens and Gelatinases (MMP2/9) which digest denatured collagens. Others include Stomelysins which activate other proMMPs, Matrilysins which process cell surface molecules and Membrane-type MMPs. Misregulation of MMP activities can lead to such conditions as cancer, arthritis or fibrosis (10). MMP transcription is regulated by cytokines, growth factors such as EGF, hormones, physical stress or oncogenic cellular transformation. Suppression of MMP

transcription occurs with glucocorticoids, retinoic acids or transforming growth factor β (TGF- β) (9). Pro-MMPs are activated by proteinases. Different tissue inhibitors of MMPs (TIMPs) help regulate action of MMPs on specific tissues for even more specialized regulation (9, 10). TIMPs bind to MMPs in a 1:1 ratio in order to inhibit MMP activity. There expression is regulated during development and tissue remodeling. TIMP levels directly affect MMP activity (10). MMP activation has been seen to be necessary for signaling in the ovary (8).

1.3 Steroid production

Steroid production in the gonads: testes and ovary, begins with gonadatropin-releasing hormone (GnRH) secretion from the hypothalamus. GnRH stimulates secretion of the gonadotropins: luteinizing hormone (LH) and follicle stimulating hormone (FSH) from gonadotrophs in the pituitary. When LH or FSH stimulate their respective receptor, this leads to increased cAMP. The Steroidogenic acute regulatory protein (StAR) is needed to bring cholesterol from the outer into the inner mitochondrial membrane to be converted to steroids (11). There are several known regulators of StAR. Dosage-sensitive sex reversal, adrenal hypoplasia congenital, critical region on the X chromosome, gene-1 (DAX-1) is known to negatively regulate StAR and therefore steroidogenesis (12). Positive regulators of StAR include: steroidogenic factor-1 (SF-1), CCAAT/enhancer binding proteins (C/EBP), steroid regulatory element binding protein (SREBP), GATA-4, Sp-1 and cyclic AMP response element binding protein (CREB) (13). Once cholesterol is in the inner mitochondrial membrane, it can then be converted to the steroid precursor pregnenolone by the enzyme CYP11A1 which can then be converted to progesterone by 3βHSD and next to androstendione by CYP 17 and subsequently to testosterone. Finally, androgens can be converted to estrogens by CYP19 (Figure 1.1). Proper steroid production is necessary for proper male and female development and fertility.

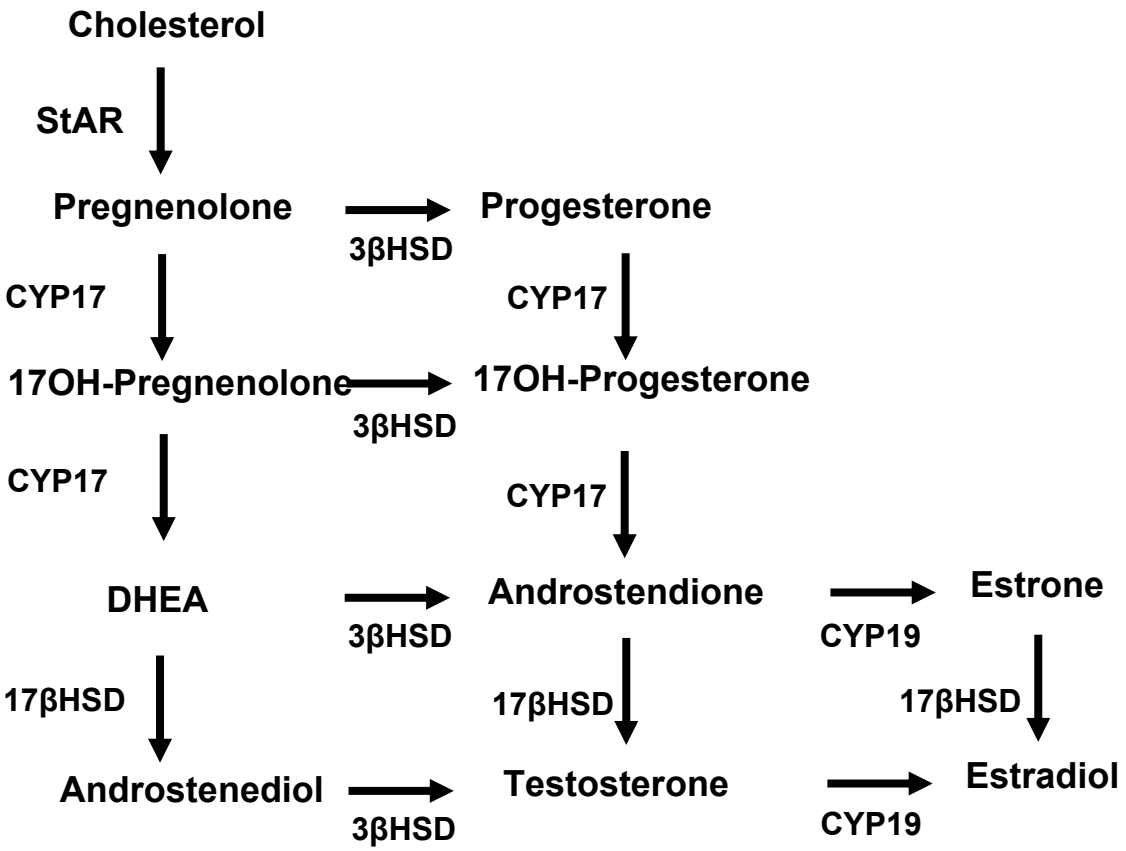

Figure 1.1. **Steroidogenic Pathways**. Cholesterol is brought into the inner mitochondria by StAR where it is then converted to the steroid precursor Pregnenolone. Then Prenenolone can be converted to Progesterone by 17βHSD. Next CYP17 converts Progesterone to 17OH-Progesterone and then Androstendione and then Testosterone. Testosterone can be converted to Estradiol with CYP19. Androstendione can also be converted to Estrone with CYP19 and subsequently to Estradiol with 17βHSD.

The Testes

The testes contain two major cell types: Sertoli cells and Leydig cells. The Sertoli cells have FSH receptors and respond to FSH to allow for proper sperm maturation. The Leydig cells are the major source of sex steroid production and have LH receptors which are G-protein coupled. LH stimulation leads to testosterone production in the testes. MMPs have not been seen to be essential for steroid production using MA-10 cells, but MMP activation may still be occurring to lead to steroid production in the testes even if it is not essential (8). MMP activation has been seen to be important for phosphorylation of the EGFR in MA-10 cells and EGFR signaling could be part of the pathway leading to steroid production in the testes as expanded on below (14).

Signals regulating steroid production in the testes

LH works through its receptor (the LHR) which is generally coupled to a Gαs protein. cAMP is activated by Gαs signaling and this leads to direct downstream activation of PKA which eventually leads to steroid production in the testes (15, 16). StAR is known to be the rate limiting step in steroid production (11, 17). Increases in PKA leads to phosphorylation of StAR and steroid production (18). Signals downstream of cAMP and before StAR are not well characterized yet. In the testes both Gαi as well as Gαs have been purified and are therefore present in the testes (16). Gαi signaling has been seen to lead to PL-C signaling after activation via LH (15, 18, 19). PL-C activation leads to activation of inositol -1,4,5-triphosphate (IP3) and diacylglycerol (DAG). These signals lead to intracellular calcium increase and PKC activation (16). PKC has been shown to increase total StAR protein (18). EGFR signaling could be important for steroid production in the testes as shown preliminarily in previous work from out lab using the mouse Leydig cell line MA-10 cells (8). Also, EGF had been shown to cause steroid production in Leydig cell lines (3, 20). There has been some evidence for the importance of Src signaling for phosphorylation of the EGFR, which could be important for steroid

production (14). Src activation can lead to activation of MAPK or MAPK can be activated independent of Src. MAPK itself has been implicated to be important for steroid production in the testes. When MAPK was inhibited, steroid production was decreased in Leydig cells but, in other studies StAR was seen to be enhanced when MAPK was inhibited (21, 22). Further studies are needed to provide better understanding of this pathway.

The Ovary

The ovary also has both LH and FSH receptors. Again, GnRH is secreted from the hypothalamus which causes release of LH and FSH from gonadotrophs in the pituitary. In the ovary there are several cell types surrounding the oocyte that allow for steroid production. Surrounding the oocyte are the cumulus granulosa cells, the mural granulosa cells and then the outer theca cells (Figure 1.2). Theca cells and the outer mural granulosa cells have both LHRs and CYP17 whereas the inner cumulus granulosa cells have FSHRs and CYP19. LHR stimulation on the theca cells eventually leads to estradiol production from the inner cumulus granulosa cells through extracellular signaling. CYP17 converts progesterone to androgens and then CYP19 converts androgens to estrogens (Figure 1.1). There is evidence that granulosa cells alone can also produce testosterone (unpublished data Hammes lab: Michelle Jamnongjit Ph.D.). Therefore, the receptors and enzymes may be more ubiquitously expressed than previously thought.

In contrast to the testes, MMPs have been shown to be essential for the process of steroidogenesis in the ovary (8). This is probably due to the need for step-wise signaling in the ovary from the outermost cells into the oocyte itself. Since the testes contain only have one major steroidogenic cell type, step-wise signaling via MMPs would not be necessary. More studies need to be done to determine which MMPs are important for the process. TNF-α converting enzyme (TACE) also known as ADAM-17 or amphireulin/epiregulin regulation could also be important (10).

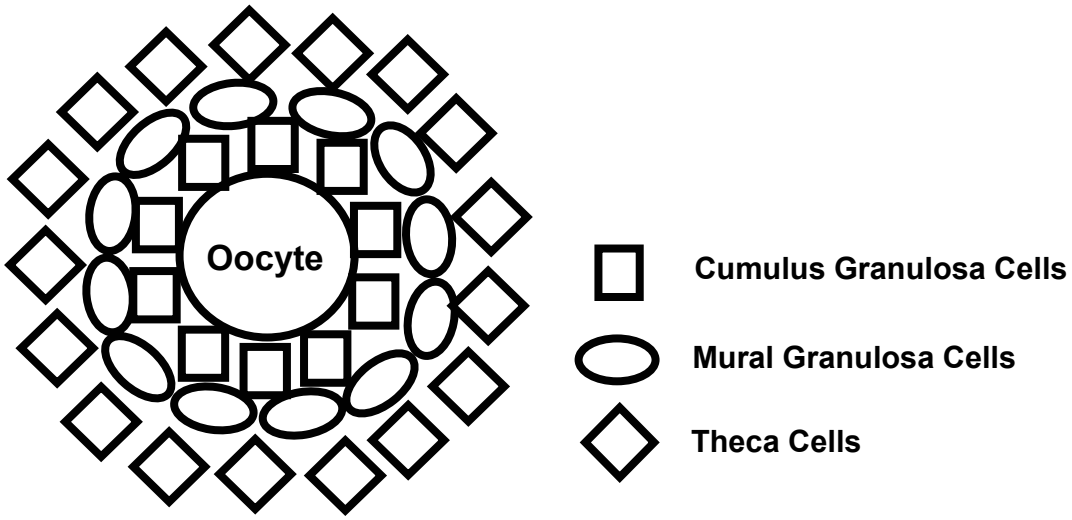

Figure 1.2. **Cells Surrounding the Ovary.** The oocyte is surrounded by cumulus granulosa cells, mural granulosa cells and finally the outer theca cells. The layers of cells allow for step-wise signaling leading towards the oocyte.

Signals regulating steroid production in the ovary

In the ovary, cAMP and PKA are also known to be important (16, 23, 24). StAR has been found to be expressed in human ovary and is the rate-limiting step for steroid production in the ovary as well (25-27). EGFR signaling has been shown to be important for steroid production. EGF can cause steroid production in two different follicle systems in the mouse ovary (8). Signals downstream of the EGFR still have not been explored. PL-C could be important working through Gαi. Again, PL-C would lead to IP3 and DAG activation which would lead to PKC activation (16, 19). MAPK and Src signaling could also be important to allow for increased steroid production. Strangely, some studies have shown that Src actually leads to a decrease in CYP17 in the ovary instead of an increase (28). This again shows a discrepancy that needs to be looked into with more experiments.

1.4 Oocyte maturation

An oocyte is arrested in an immature form in prophase of meiosis I until the presence of steroid allows for resumption into meiosis. This resumption into meiosis is referred to as oocyte maturation. Next, the oocyte is arrested in metaphase II as a mature oocyte ready for ovulation and subsequent fertilization. The process of oocyte maturation is transcription independent. When Actinomycin-D is used to inhibit transcription, maturation still occurs (29-31). Oocytes are held in meiotic arrest due to constitutive G-protein coupled receptor 3 (GPR3) activity which has been shown to signal via Gβγ. This allows for cAMP levels to be kept high (32-35). Release of inhibition and subsequent maturation occurs when steroid is produced as due to signaling pathways activated by LH activating its receptor, as previously described. Once steroid is present, cAMP is decreased and signals holding the oocyte in arrest are downregulated so that maturation can occur. This overall process is illustrated in Figure 1.3.

Which steroid is causing oocyte maturation in the mouse?

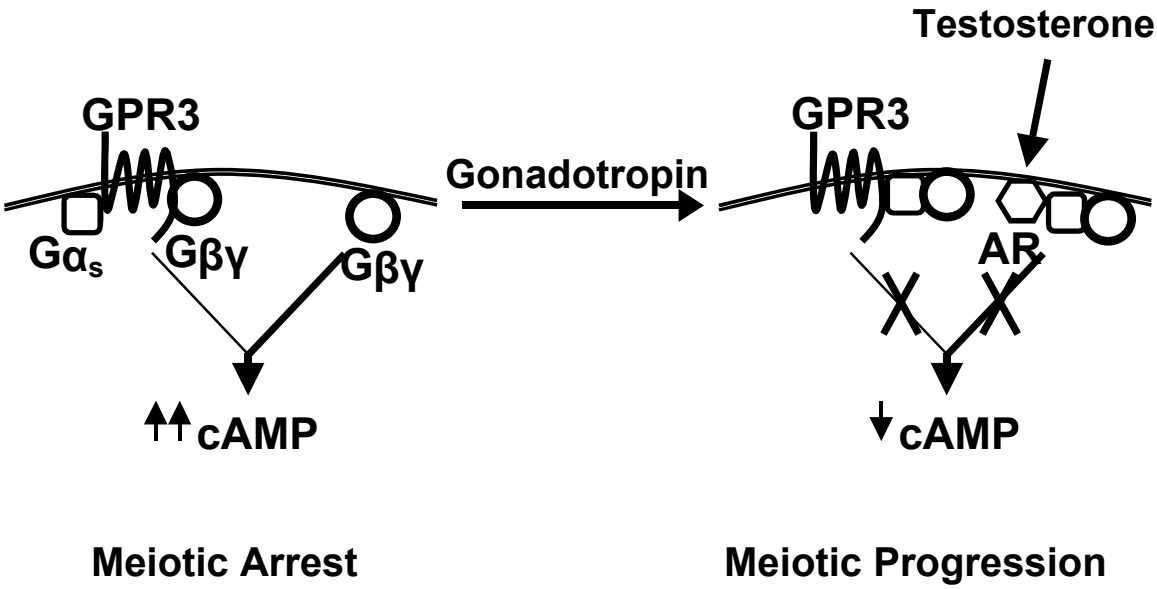

Figure 1.3. **Release of Inhibition Model for Oocyte Maturation.** Prior to the presence of steroid, the oocyte is arrested in an immature state. GPR3 keeps the oocyte in meiotic arrest via Gβγ signaling specifically, which keeps cAMP high. Both Gα and Gβγ signaling occurs, but Gβγ seems to be more important for this process. After gonadotropin induced steroid production, Gβγ signaling is interrupted and cAMP is decreased to allow for meiotic resumption.

In mice, testosterone added *in vitro* was seen to cause activation of signals that lead to oocyte maturation, such as phoshorylation of MAPK, specifically in the oocyte. Testosterone also caused oocyte maturation *in vitro*. Addition of the classical AR antagonist, Flutamide, decreased testosterone mediated-phosphorylation of MAPK by 50-70%. This showed the classical AR to be partially important for this process in mice. Estradiol was also seen to increase P-MAPK and oocyte maturation *in vitro*. When estradiol signaling was blocked using the classical estrogen receptor (ER) antagonist ICI 182,780, estradiol induced-P-MAPK was decreased. Therefore, the classical ER may be important for oocyte maturation in mice also. Progesterone, added *in vitro,* increased P-MAPK in the oocytes, which implicates progesterone as a possible promoter of oocyte maturation in mice as well (31). Other evidence showed that blocking FSH-induced maturation could be partially rescued by adding progesterone *in vitro* using mouse cumulus cell complexes (COCs) (36). It has recently been shown that testosterone can cause MAPK activation and oocyte maturation in another mammalian model, porcine oocytes. Flutamide blocked oocyte maturation in porcine oocytes, supporting the idea that the classical androgen receptor is important for the process of oocyte maturation in mammals (37).

Which steroid is causing oocyte maturation in the frog?

Although other mammals are more closely related to humans, we have used the *Xenopus laevis* frog model for the following experiments dealing with oocyte maturation. *Xenopus laevis* oocytes are easy to genetically manipulate by RNA microinjection and are therefore ideal for discovering more about the signals needed to keep oocytes in meiotic arrest as well as the signals needed to overcome meiotic arrest. It is also very easy to differentiate a mature versus an immature *Xenopus* oocyte making this model even more amenable to our studies.

For many years progesterone was used to induce maturation of *Xenopus laevis* oocytes *in vitro*. We found that testosterone was just as potent *in vitro*. Also, we showed that testosterone is actually the physiologic mediator in *Xenopus laevis in vivo*. Serum levels of testosterone were

high in the whole frog, whereas barely any progesterone was present. When added to isolated oocytes, progesterone is rapidly metabolized to androstendione by endogenous CYP17 (38). This means adding progesterone is essentially the same as adding testosterone. Therefore, in our lab, we use testosterone to promote maturation for *Xenopus laevis* oocytes. Testosterone action to allow for oocyte maturation is shown in Figure 1.3.

Which receptor is steroid-induced maturation occurring through? It is possible that the classical androgen receptor (AR) or progesterone receptor (PR) is being brought to the membrane when ligand is available to allow for maturation. If this is the case, testosterone signaling could be specifically working though the AR and progesterone though the PR or there could be cross-talk between the two ligands and receptors that might account for why either steroid has a similar affect *in vitro* (30). There is also a possibility that the steroids are working though membrane receptors such as the membrane progesterone receptor (mPR). The exact role of mPR has been controversial. It was first isolated in fish and shown to bind to progesterone and be found exclusively in the plasma membrane (39), but later papers have shown conflicting evidence that the mPR might actually be located on the endoplasmic reticulum membrane instead of the plasma membrane. There is also some discrepancy as to whether or not the mPR actually binds progesterone (40). More studies are needed to see which receptors the steroids are acting through to allow for oocyte maturation and to tease out the underlying mechanism for why testosterone and progesterone seem to be equally potent promoters of maturation *in vivo* despite the fact that only testosterone is actually present in the whole frog.

Signals holding the oocyte in meiotic arrest

G proteins are known to be holding the oocyte in meiotic arrest (35, 41, 42). One G protein-coupled receptor, GRP3, has recently been shown to participate in holding oocytes in meiotic arrest. GPR3 is a constitutively active orphan receptor and has no known ligand (32, 43). It is possible that other G protein-coupled receptors are involved in the process as well. Both Gα

signaling and Gβγ signaling have been implicated in the process of holding oocytes in meiotic arrest, but we have seen evidence that Gβγ subunits are the main mediator. Constitutively active Gαi does not affect steroid-induced maturation, whereas rat Gαi enhanced maturation which makes it unlikely to be the main mediator. In contrast, overexpression of both Gβ and Gγ together stopped steroid-induced maturation. Also, sequestering β and γ using both tranducin Gα, which can act as a Gβγ sink, and the GRK minigene, which is the carboxyl portion of the GRK1 protein known to inhibit Gβγ, caused attenuation of maturation. Changes induced by Gαi are probably due to free Gβγ since we have shown that constitutively active Gαi, which is unable to affect free Gβγ levels, had a minimal affect on maturation (41).

High cAMP levels are necessary to keep oocytes in meiotic arrest both in mice and in *Xenopus laevis*. When the oocytes are removed from the frog, they stay in meiotic arrest with high cAMP levels (35, 41, 42, 44-46). Mouse oocytes will spontaneously mature if separated from the ovary unless cAMP levels are held artificially high with isobutylmethylxanthine (IBMX) (31). This shows that for mammals the ovary is signaling to hold the oocyte in meiotic arrest whereas in the frog the oocyte itself is providing signals to stay immature. The signals could be similar, but coming from different sources. We were able to show that overexpression of *Xenopus* GPR3 in *Xenopus* oocytes caused increased cAMP levels. Also, co-overexpression of *Xenopus* GPR3 with the GRK minigene attenuated GPR3-induced rise in cAMP reiterating the importance of Gβγ signaling (32).

The modulator of nongenomic actions of the estrogen receptor (MNAR) is possibly acting as a scaffolding protein holding the steroid receptor and G protein-coupled receptor together at the membrane of the oocyte. When MNAR was reduced by RNAi, steroid-induced maturation was enhanced. This shows MNAR to be important in holding the oocytes in meiotic arrest. Also MNAR overexpression enhanced AR-mediated transcription in CV1 cells. MNAR and AR could be co-immunoprecipitated. Also, MNAR and Gβ could be co-immunoprecipitated

and reduction of MNAR decreased Gβγ signaling. Therefore, it is possible that androgen binding may cause release of AR from MNAR and allow for release of inhibition (47).

Signals leading to maturation

In order for maturation to occur, there is a positive feedback loop which is started by polyadenylation of MOS protein, a germ-cell specific mitogen-activated protein kinase kinase (MEK) similar to Raf, which activates MEK1 and then ERK2. ERK2 works to activate the expression of more MOS to facilitate the positive feedback loop. The scaffolding protein Paxillin has recently been implicated in this process to be necessary for the accumulation of MOS protein to keep the loop going. Reducing Paxillin by RNAi or antisense oligonucleotides stops steroid-triggered oocyte maturation. Paxillin has been shown to be phosphorylated on a serine residue by a downstream MAPK signal for full activity (48). Once maturation occurs it is irreversible, so this positive feedback loop is important in ensuring that maturation occurs in one direction. Further studies are being done to see the mechanism of this action and to discover other possible players.

1.5 Polycystic Ovary Syndrome

Polycystic Ovary Syndrome (PCOS) is characterized by irregular or absent menstrual periods, lack of ovulation (anovulation), excess androgen production, polycystic ovaries, insulin resistance and frequently obesity (49, 50). Six-7% of women suffer from PCOS and it is the leading cause of infertility (51). Both inherited and environmental factors may contribute to the development of PCOS. There is controversy in the field as to whether insulin resistance or improper steroid production initiates the syndrome. It is probably a synergistic affect and either or both could be the catalyst for the PCOS phenotype. Since the pathways are linked,

dysregulation of one can easily cause a problem with the other. This makes it even harder to tease out the initial problem.

Anovulation means that one follicle, also known as a cyst, does not become dominant to allow for one egg to be released. Instead several mid-antral follicles are formed (52). There is a portion of the LH signaling pathway that we believe is required for normal ovarian steroidogenesis as described previously in this thesis. If LH signaling in the ovary is abnormal, excess androgen could be produced by the follicle. So-called "androgen dominant" follicles will not fully develop, no follicle will become dominant and the patient will become anovulatory (49, 52). As a secondary effect, abnormal steroid feedback at the hypothalamus increases GnRH pulse frequency and therefore increases LH secretion, further stimulating elevated androgen production in the ovary (51). Consistent with the proposed role of androgens in this disorder, targeting androgens specifically by using AR antagonists seems to help with infertility for some PCOS patients (49, 51). Proper regulation of at the steps of MMP activation or EGFR signaling in the ovary may be important for proper steroid production and could also be possible targets for drugs as will be discussed in the Chapter 4 Conclusions and Future directions.

Also of interest, 50-70% of PCOS patients are insulin resistant and later develop hyperinsulinemia. The obesity present in a significant number of patients undoubtedly contributes to the insulin resistance; however, lean patients with PCOS may also be insulin resistant suggesting an inherited defect. Hyperinsulinemia has also been correlated with high circulating androgen levels and anovulation. Insulin can cause an increase in ovarian steroid production. Low-density lipoprotein (LDL) is the major source of cholesterol needed for steroid-biosynthesis and is transported into the ovary via the LDL receptor pathway. LH and insulin can work together to cause increase in LDL receptor protein and gene expression and subsequent transcriptional activity. LH and insulin caused increase in StAR and CYP17 which would increase steroid production. Reducing insulin levels by causing more sensitivity to insulin has helped lower hyperandrogenemia and allow for ovulation in some patients (53). These studies

provide additional insight into mechanisms regulating normal ovarian steroid production and may ultimately allow for better understanding of what has gone wrong in PCOS, suggesting alternate treatments for this common disorder.

Chapter 2

Signaling Pathway for Steroid Production in the Testes

Introduction

Steroid production in the testes begins with gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus. GnRH stimulates pulsatile release of luteinizing hormone (LH) from gonadotrophs in the pituitary, followed by LH binding to G protein-coupled LH receptors on testicular Leydig cells to promote steroidogenesis. In males, LH pulsations occur approximately every two hours, and this steady rhythm is felt to be important for maximum testosterone production (54, 55).

In Leydig cells, LH-induced cAMP production is a critical regulator of steroid production (56-59). One of the major mechanisms by which cAMP promotes steroidogenesis is by increasing expression of the Steroidogenic Acute Regulatory Protein (StAR) (27, 60, 61). StAR is needed to bring cholesterol into the mitochondria for conversion to steroid, an event generally believed to be the rate-limiting step in steroid production. Interestingly, evidence suggests that phosphorylation of StAR is critical for its activation and translocation from the cytoplasm to the mitochondria (62).

In addition to cAMP, several studies have implicated Epidermal Growth Factor Receptor (EGFR) signaling as a potential regulator of steroidogenesis in both the ovary and testes. First, EGF increases StAR expression in Leydig cells over the course of several hours (3, 63). Second, human chorionic gonadotropin (hCG) triggers rapid phosphorylation of the EGFR in MA-10 mouse Leydig cells that are over-expressing LH and EGF receptors (14, 64). Finally, inhibition of EGFR signaling blocks LH-induced steroid production in MA-10 Leydig cells, as well as in isolated ovarian follicles (8).

The mechanism by which LH receptor signaling triggers activation of the EGFR is still controversial. Several *in-vitro* studies of other G protein-coupled receptors (GPCRs) have shown that the GPCRs can trans-activate EGFRs through matrix metalloproteinase (MMP)-mediated release of membrane-bound EGFR-activating ectodomains (e.g., HB-EGF, amphiregulin, and epiregulin) (65-68). In contrast, other studies suggest that such EGFR trans-activation can occur independent of MMPs through intracellular signaling pathways that might include cAMP and/or Src (69, 70). In mouse follicles, MMP inhibitors block EGFR phosphorylation, gonadotropin-induced oocyte maturation, and steroidogenesis, suggesting that extra-cellular signaling is essential for EGFR trans-activation. (8, 71, 72). In MA-10 mouse Leydig cells, MMP inhibitors also reduce phosphorylation of the EGFR (14, 64). However, this reduction in the Leydig cells is only partial, and MMP inhibition does not block gonadotropin-induced steroidogenesis in the same cells (8). Therefore, the importance of MMPs in regulating LH actions in the testes remains uncertain.

To address the role of LH and EGF receptor cross-talk in the physiologic response to gonadotropin signaling in Leydig cells, steroid production and release, we performed detailed signaling and steroidogenesis studies in the mouse MLTC-1 Leydig cell line. These cells express endogenous LH and EGF receptors, and rapidly produce progesterone in response to LH or hCG stimulation. We found that LH receptor activation led to rapid but transient cAMP-dependent activation of the EGFR and downstream Mitogen-Activated Protein Kinase (MAPK) cascade. This gonadotropin-induced kinase cascade was essential for short-term (approximately 30 minutes), but not prolonged (approximately 2 hours), LH receptor-mediated steroidogenesis. Importantly, both short- and long-term LH-induced steroidogenesis occurred independent of MMP activation, suggesting that, in Leydig cells, the EGFR pathway was activated through intra- rather than extra-cellular signals.

Materials and Methods

Tissue Culture and Materials

MLTC-1 mouse Leydig cells (ATCC) were maintained in RPMI 1640 medium (Fisher Scientific) supplemented with 10 mM Hepes, 5% Pent Strep (Invitrogen) and 10% Fetal Bovine Serum (Invitrogen). For each study, cells were plated in either 12- or 6-well plates followed by serum starvation overnight to synchronize the cells and reduce background kinase activity. Notably, most of the steroidogenesis experiments were also performed without serum starvation and the results were identical. Galardin, TAPI-1, AG1478, H-89, PP2 and U0126 (Calbiochem) were added for 30 minutes prior to hCG (Sigma) stimulation, and kept present throughout each experiment. Erlotinib was a gift from Dr. John Minna (UT Southwestern). Forskolin was from Calbiochem and FGF-2 from PeproTech.

EGFR/MAPK Activation

After treatment with hCG +/- inhibitors as indicated in the figure legends, cells were washed once with cold PBS followed by addition of 125 µl NP40 lysis buffer (0.5% NP-40, 50 mM Tris-Hcl, pH 8.0, 150 mM NaCl, 1mM EDTA, 2 mM NaF, 0.5 mM NaVanadate, 10 mM Sodium Phosphate, pH 7.4). The lysis buffer was supplemented with 10 mcg/ml Pepstatin A (Sigma), 10 mcg/ml Leupeptin (Sigma), 10 mcg/ml Aprotinin (Sigma) and 100 mcg/ml phenylmethylsulfonyl fluoride. Plates were placed on a shaker at 4 °C for 15 minutes, cells were scraped and microcentrifuged at full speed for 15 minutes, and supernatants removed and diluted 1:2 in 2x Laemmli sample buffer with 10% ß-mercaptoethanol (Sigma-Aldrich). Western blots for phospho- and total-EGF receptor and extra-cellular signal-related kinase (ERK)1/2 were performed as described below.

StAR Expression

Mitochondrial isolation was performed as described (3). Briefly, hCG +/- inhibitors were added to cells in 6-well plates for the indicated times, cells were washed one time with cold PBS and then placed in 250 µl TSE Buffer (0.25 M sucrose, 10 mM Tris-HCL, pH 7.4, 0.1 mM EDTA). After 15 minutes of gentle shaking in the cold room, the cells were scraped from the plates and homogenized using 20 strokes of a pellet pestle (Kimble Kontes). Lysates were then centrifuged at 600 x g for 15 minutes at 4°C. Finally, supernatants were centrifuged 10,000 x g for 15 minutes at 4°C, the mitochondrial pellets were re-suspended in 60 µl TSE, and samples were diluted 1:2 in 2X Laemmli sample buffer with 10% β-mercaptoethanol (Sigma-Aldrich). The BCA protein Assay Kit (Thermo Scientific) was used to measure protein concentrations, and equal amounts of protein were analyzed by SDS-polyacrylamide gel electrophoresis followed by Western blot analysis as described below.

Primary Leydig Culture

All mice were treated in accord with accepted NIH and University of Texas standards of humane animal care. The primary Leydig cell culture protocol was adapted from Hamra et al (73). Briefly, 2-4 month old male mice were used. The testes were removed and added to 20 mL DMEM:HamF12 (1:1) (Sigma) medium containing 0.5 mg/ml Collagenase 1A (Sigma) and 1.0 mg/ml soy bean Trypsin inhibitor (Sigma). Samples were incubated at 37°C for 20 minutes while rocking by hand every 5 minutes. After allowing the pellets to settle by gravity for a few minutes, supernatants were filtered through 70-micron mesh (BD Falcon) twice. The filtrates were centrifuged for 5 min at 200 x g, supernatants removed, and cellular pellets re-suspended in DMEM:HamF12 (1:1) medium containing 2% FBS. Cells were placed in 6-well plates and stimulated with hCG +/- inhibitors for steroidogenesis as described.

Western Blot

Samples were separated on 7.5% SDS-polyacrylamide gels for EGFR detection, 10% gels for ERK1/2, or 14% gels for StAR. Proteins were then transferred to Immobilon membranes (Millipore Corp., Billerica). These membranes were blocked with 5% milk (Carnation) in TBST (100 mM NaCl; 0.1% Tween 20; 50 mM Tris, pH 7.4) for 1 hour and incubated overnight at 4° C with primary antibody in TBST plus 5% milk. Blots were then washed three times with TBST, incubated for 1 hour at room temperature with (1:4000) horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody (Bio Rad) in TBST plus milk, and washed another three times with TBST. Finally, blots were treated with ECL-Plus (GE Healthcare) to visualize the proteins. For EGFR detection, 1:2000 anti-phospho-EGFR Tyr1173 (Upstate Cell Signaling Solutions) and 1:1000 anti-total-EGFR (Cell Signaling) were used. For ERK signaling, 1:2000 anti-phospho-ERK1/2 or anti-total-ERK1/2 (Cell Signaling) antibodies were used (41). For StAR detection, 1:10,000 anti-StAR antibody (Doug Stocco, Texas Tech Health Sciences Center, Lubbock, TX) and 1:2000 anti-phospho-StAR antibody (Dr. Steven King, Baylor College of Medicine, Houston TX) were used. For StAR detection, 30 mcg of total mitochondrial protein was used per well.

Steroid Radioimmune Assays

To measure steroid content in the media, a Progesterone RIA kit (MP Biomedicals) was used for MLTC-1 cells, while a testosterone RIA kit (MP Biomedicals) was used for the primary Leydig cells. Assays were performed as directed in the inserts.

***In-vivo* Steroidogenesis Assays**

Seven week old male C57/B6 mice were injected IP with either 20 μM AG1478 suspended in 100 μl phosphate-buffered saline (0.1% DMSO) or PBS/DMSO alone. Injections were repeated after 24 hours, and serum removed from anesthetized mice by cardiac puncture eight hours after the second injection. Serum testosterone levels were measured by RIA.

Results

EGFR Signaling is necessary for short- but not long-term gonadotropin-induced steroidogenesis.

To determine the sensitivity of MLTC-1 Leydig cells for gonadotropin-induced steroidogenesis, cells were treated with increasing amounts of hCG for 30 minutes and progesterone levels in the media were measured by RIA. As shown in Fig. 2.1A, the EC50 for hCG-induced steroidogenesis was approximately 0.05 IU/ml, with saturation occurring at about 0.5 IU/ml. Saturating amounts of hCG (5 IU/ml) were used for all studies shown in this manuscript, although similar results were obtained when using concentrations near the EC50 (data not shown). Importantly, hCG-induced steroid production was consistently detectable as early as 30 minutes, and reached a steady peak at about two hours (Fig. 2.1B). Therefore, for all studies in this manuscript, steroid production was measured at both 30 and 120 minutes.

To determine the importance of EGFR signaling in gonadotropin-induced steroid production, MLTC-1 mouse Leydig cells were serum starved overnight and pretreated for 30 minutes with the EGFR inhibitors AG1478 or Erlotinib. Cells were then incubated with hCG for 30 minutes or two hours with the inhibitors still present. EGFR signaling was required for hCG-induced steroidogenesis at 30 minutes, as both AG1478 and Erlotinib almost completely abrogated steroid production (Fig. 2.2A, open bars). Surprisingly, neither of the EGFR inhibitors significantly blocked hCG-induced steroidogenesis at 2 hours (Fig. 2.2A, closed bars), suggesting that EGFR signaling was no longer required for steroidogenesis at the later time point. Accordingly, gonadotropin-induced EGFR activation was only seen at the early time point, as hCG induced significant phosphorylation of the EGFR at 30 minutes, but virtually none at 2 hours (Fig. 2.2B). This loss of EGFR activation in the setting of persistent steroidogenesis suggested that alternative pathways were being utilized to promote steroid production at two hours. Notably, both AG1478 and Erlotinib abrogated hCG-induced phosphorylation of the

EGFR at 30 minutes and baseline EGFR phosphorylation at 2 hours (Fig. 2.2B), confirming that they were inhibiting EGFR kinase activity as expected and for the duration of the experiment.

Interestingly, EGF alone at a concentration that trigger phosphorylation of the EGFR (data not shown) did not promote significant steroid production at either 30 minutes or two hours (Fig. 2.2C), indicating that EGFR signaling is necessary but not sufficient for early gonadotropin-induced steroidogenesis in MLTC-1 cells.

How is the EGFR being activated in response to hCG? Some studies demonstrate that GPCR-mediated activation of MMPs can promote the shedding of EGFR ligands from the cell surface that then act in an autocrine/paracrine fashion to activate EGFRs (65-68). In fact, recent studies suggest that MMP activation may contribute to LH-induced activation of the EGFR in MA-10 Leydig cells (14, 64), although MMP inhibition did not appear to affect LH-induced steroid production in the same cells (8).

In MLTC-1 mouse Leydig cells, MMP activation was not required for hCG-induced steroidogenesis at any time point, as the broad-spectrum MMP inhibitor Galardin had no effect on steroidogenesis (Fig. 2.2A). Notably, this same batch of Galardin could inhibit hCG-induced steroid production in ovarian follicles (data not shown), where MMP activation is clearly important for steroidogenesis (8), indicating that the inhibitor was functioning appropriately. Furthermore, the TNF-α converting enzyme (TACE) inhibitor, TAPI-1, had no effect on hCG-induced steroidogenesis in MLTC-1 Leydig cells (data not shown), indicating that, in addition to MMPs, TACE-mediated release of EGFR-activating ectodomains was not regulating gonadotropin-induced steroidogenesis.

Finally, to confirm the aforementioned results in a more biologically relevant setting, primary Leydig cells were isolated from adult male mice and similarly treated with hCG and an EGFR inhibitor. Similar to the results in the MLTC-1 Leydig tumor cell line, the EGFR inhibitor AG1478 significantly blocked steroid production at 30 minutes but not at 2 hours in primary Leydig cells (Fig. 2.2D).

Importantly, while steroidogenesis in primary Leydig cells was not altered by AG1478 at two hours using an *in-vitro* assay, *in-vivo* treatment of male mice with AG1478 for 36 hours significantly reduced serum testosterone levels relative to mock-injected mice (Fig. 2E), confirming the physiologic importance of LH and EGF receptor cross talk in regulating testicular steroidogenesis.

EGFR signaling activates the MAPK cascade to regulate gonadotropin-mediated steroid production.

To determine the role of other intracellular kinases in the regulation of gonadotropin-induced steroidogenesis, the effects of both Src and MEK inhibitors on hCG-mediated steroid production were examined. In some systems, Src kinases have been implicated as potential regulators of GPCR-mediated trans-activation of the EGFR (65). However, the Src inhibitor PP2 had no effect on hCG-induced steroid production at either 30 minutes or 2 hours in MLTC-1 cells (Fig. 2.3A), indicating that Src signaling is not necessary for steroid production in Leydig cells. In contrast, similar to the results using EGFR inhibitors, blockade of MAPK kinase (MEK) signaling with U0126 almost completely eliminated steroidogenesis at 30 minutes, but had significantly less of an affect at 2 hours (Fig. 2.3B). As with EGFR signaling, MEK activity was dramatically induced by hCG at 30 minutes but was barely present at two hours (Fig. 2.3C). Notably, U0126 abrogated any detectable MEK activity at both time points, confirming that is was functioning appropriately throughout the time course of the experiment. These findings suggested that, like the EGFR, MEK signaling was important for early, but not late, gonadotropin-induced steroid production. Furthermore, the nearly identical patterns of EGFR and MEK signaling suggested that these two kinases may have been functioning together during early gonadotropin-induced steroid production. In fact, while U0126 blocked MEK activity at 30 minutes (Fig. 2.3C), it had no effect on hCG-induced EGFR activation (Fig. 2.3D). In contrast, the EGFR kinase inhibitor AG1478 significantly blocked both EGFR activation (Fig. 2.2A) and

MEK signaling (Fig, 2.3E, third lane). These results indicate that MEK signaling is likely downstream of EGFR signaling.

Importantly, addition of fibroblast growth factor (FGF) rescued the inhibitory effects of AG1478 on hCG-induced MEK activation (Fig. 2.3D, fourth lane), confirming that AG1478 was not directly blocking MEK signaling. However, FGF did not rescue steroidogenesis under these conditions (Fig. 2.3F, fourth bar). Furthermore, as seen with EGF (Fig. 2.2C), MEK activation by FGF alone was not sufficient to promote steroidogenesis at 30 minutes (Fig. 2.3F, last bar). Together, these results suggest that activation of both the EGFR and MEK are necessary, but not sufficient, for early gonadotropin-induced steroidogenesis in Leydig cells.

cAMP and Protein kinase A (PKA) are required for activation of the EGFR during early gonadotropin-induced steroid production.

Since cAMP and PKA are known to be critical regulators of steroid production in all steroidogenic tissues (56-59), the role of these signaling molecules in regulating steroidogenesis in the context of EGFR trans-activation was examined. As expected, inhibition of PKA signaling with H-89 significantly reduced hCG-induced steroidogenesis in MLTC-1 cells at both 30 minutes and 2 hours (Fig. 2.4A). Interestingly, H-89 abrogated hCG-induced phosphorylation of both the EGFR and ERK1/2 at 30 minutes (Fig. 2.4B & C), suggesting that PKA acted upstream of EGFR signaling. Notably, as seen in Figures 2 and 3, hCG-induced phosphorylation of the EGFR and MEK were significantly reduced at 2 hours (Fig. 2.4B & C). Furthermore, H-89 completed abrogated the small amount of remaining hCG-induced MEK activity at 2 hours (Fig. 2.4C), confirming that the drug was still working at the later time point.

To determine whether cAMP and PKA signaling were sufficient to promote activation of the EGFR and downstream MEK, MLTC-1 cells were treated with 10μM forskolin, which stimulates adenylyl cyclase to increase intracellular cAMP levels. This concentration of forskolin was chosen because it promotes similar levels of steroid production as 5 IU/mL hCG. As

expected, forskolin promoted significant steroid production at both 30 minutes and 2 hours (Fig. 2.4D). This forskolin-induced steroidogenesis was PKA dependent, as H-89 completely abrogated steroid production at both time points (Fig. 2.4D). Interestingly, similar to hCG stimulation, blockade of EGFR signaling (using Erlotinib) or MEK activity (using U0126) almost completely eliminated forskolin-induced steroidogenesis at 30 minutes, but not two hours. Furthermore, Erlotinib blocked forskolin-induced MEK activation (Fig. 2.4F), while U0126 had no affect on forskolin-mediated EGFR activation (Fig. 2.4E). Together, these results indicated that cAMP/PKA signaling was both upstream of and sufficient to activate EGFR and subsequent MEK signaling, as well as to promote short-term steroidogenesis.

EGFR signaling may be regulating steroidogenesis by activating StAR

As mentioned, StAR is an important rate-limiting protein in gonadotropin-induced steroid production, and some evidence suggests that StAR may be regulated by EGFR signaling in gonadal tissues (3, 63). To address the relationship between EGFR signaling and StAR activity in MTLC-1 Leydig cells, the effect of the EGFR inhibitor AG1478 on StAR-independent steroidogenesis was examined. Cells were treated for 30 minutes with 22R-cholesterol, which can enter the mitochondria and serve as a substrate for steroidogenesis independent of StAR. 22R-cholesterol stimulated significant steroid production that was unaffected by AG1478 (Fig. 2.5A), implying that EGFR signaling was not necessary for steroidogenesis if StAR is bypassed. Interestingly, hCG promoted significant mitochondrial translocation of total StAR that was first detectable at one hour (Fig. 2.5B). Similar to its effects on steroidogenesis, the EGFR inhibitor AG1478 significantly blocked StAR translocation to the mitochondria at the early time point, but not at two hours (Fig. 2.5B). This result was much more dramatic when blotting for phosphorylated StAR (Fig. 2.5B, bottom). As expected, inhibition of MEK with U0126 similarly reduced hCG-induced translocation of StAR to the mitochondria at one hour (data not shown). Together, these findings suggested that hCG-mediated activation of the EGFR might promote

phosphorylation of StAR and translocation to the mitochondria at early time points; however, EGFR/MEK signaling may be less important for StAR activation at 2 hours or later.

Discussion

Many *in-vitro* examples of cross-talk between G protein-coupled and EGF receptors have been described (65-68). Interestingly, the mechanisms regulating this cross-talk vary depending upon both the GPCR as well as the cell type. For example, in COS cells, β2-adrenergic receptor activation triggers activation of the EGFR in an intracellular, ligand-independent, process that may involve intracellular Src signaling (69, 74). In contrast, the β2-adrenergic receptor activates the EGFR in an extra-cellular, ligand-dependent, fashion in cardiac fibroblasts by stimulating matrix metalloproteinases (MMPs) to cleave membrane-bound EGFR ligand ectodomains (68). As another example, LPA trans-activates the EGFR via MMPs and ectodomain shedding in COS cells (67), but through intracellular mechanisms in adrenal glomerulosa cells (70).

While *in-vitro* studies of GPCR/EGFR cross-talk have been beautifully characterized, the *in-vivo* importance of this trans-activation is still under investigation. Recent studies in the gonads suggest that MMP-mediated release of EGFR-activating ectodomains from amphiregulin and epiregulin are important for mouse oocyte maturation (71), as well as LH-induced steroidogenesis in ovarian follicles (8). Gonadotropin-mediated activation of the EGFR may also regulate steroidogenesis in Leydig cells; however, the mechanism by which the LH receptor activation initiates EGFR signaling in the testes remains controversial. For example, both Src and MMPs may play partial roles in rapidly trans-activating the EGFR MA-10 mouse Leydig cells that are over-expressing LH and EGF receptors (14, 64). However, MMP inhibition does not appear to be critical for LH-induced steroid production in native MA-10 cells (8).

Here we have attempted to reconcile some of the controversies regarding LHR/EGFR cross-talk in Leydig cells. Rather than use partial changes in intracellular signaling as our endpoint, we instead focused on the physiologically important endpoint of gonadotropin-induced steroid production. In addition, to avoid complications due to over-expression of receptors, we used native MLTC-1 mouse Leydig cells that express only endogenous LH and EGF receptors.

We found that the EGFR and MEK were activated after 30 minutes stimulation with hCG, with MEK signaling occurring downstream of EGFR activation (Fig. 2.2). Furthermore, EGFR and MEK activation were essential for normal hCG-induced steroidogenesis at 30 minutes. Similar to previous reports (14, 64), we found that MMP inhibition partially blocked hCG-induced EGFR and MEK activation (data not shown). However, MMP inhibitors, including Galardin (Fig. 2.2A) and the TACE inhibitor TAPI-1 (data not shown), had no effect on the final physiologic endpoint of steroidogenesis, suggesting that intracellular, as well as MMP-dependent extra-cellular, activation of the EGFR must be occurring. These observations differ from those in ovarian follicles, where Galardin markedly inhibited LH-induced steroid production (8). To explain these two results, we propose that LH receptor activation may trigger EGFR trans-activation through both extra-cellular (MMP-mediated) and intracellular (MMP-independent) mechanisms in both the ovaries and testes. However, the extra-cellular pathway may be less important in the testes, where all steps of steroidogenesis occur within individual Leydig cells. In contrast, the gonadotropin-induced extra-cellular pathway might be essential in the ovarian follicle, where paracrine signaling is required for communication between theca and mural granulosa cells, which express LH receptors, and cumulus granulosa cells, which lack LH receptors

If MMPs are not involved, then what signals are being triggered by the LH receptor to trans-activate the EGFR in Leydig cells? As mentioned, Src has been implicated in rapid EGFR trans-activation; however, simlar to the MMP inhibitors, we found that inhibition of Src with PP2 had no affect on the biologic endpoint of gonadotropin-mediated steroidogenesis (Fig. 2.3A). Instead, PKA inhibition completely blocked hCG-induced EGFR and MEK activation, as well as steroidogenesis, at 30 minutes (Fig. 2.4). Furthermore, stimulation of cAMP production with forskolin for 30 minutes promoted steroidogenesis in an EGFR- and MEK-dependent fashion. These observations indicate that cAMP and PKA are both necessary and sufficient for short-term EGFR-mediated steroidogenesis. Interestingly, cAMP/PKA signaling may be a conserved

mechanism for regulating LH and EGF receptor cross-talk, as cAMP appears to mediate gonadotropin-induced trans-activation of the EGFR during oocyte maturation (72). Whether PKA directly or indirectly activates the EGFR is still unclear; however, PKA may be able to directly phosphorylate and activate the EGFR under some conditions (75, 76).

Interestingly, LHR/EGFR cross-talk occurred at early (30 minutes), but not late (120 minutes) time points (Fig. 2.2). Accordingly, two different EGFR inhibitors (AG1478 and Erlotinib), as well as the MEK inhibitor U0126, blocked gonadotropin-induced steroidogenesis at 30, but not 120, minutes. Together, these data indicate that the EGFR/MEK kinase cascade is essential for short-, but not long-term gonadotropin-induced steroidogenesis.

How might the EGFR/MEK kinase cascade be regulating short-term gonadotropin-induced steroidogenesis? The answer may lie in part with StAR, which is the rate-limiting regulator of steroidogenesis in all steroidogenic tissues. Interestingly, phosphorylation of StAR at residue 194 appears to be important for its activation (62). As with steroidogenesis, short (30 minutes)-, but not long (2 hours)-term hCG-induced phosphorylation and translocation of StAR to the mitochondria was significantly reduced by the EGFR inhibitor AG1478 (Fig. 2.5). Thus, we propose that the EGFR/MEK kinase pathway is important for early phosphorylation and translocation of StAR, when StAR levels are limiting, but may not be needed at 2 hours, when StAR protein levels are elevated due to cAMP-mediated gene transcription and subsequent translation.

What is the biological importance of this bi-modal activation? In humans, the pituitary releases LH every 90-120 minutes, with serum concentrations varying as much as 2-fold (77, 78). Thus, the EGFR/MEK kinase pathway may be important for chronic androgen production *in vivo*, as Leydig cells might be constantly re-sensitizing to LH and therefore re-activating the kinase cascade. Consistent with this interpretation, treatment of male mice with AG1478 for 36 hours significantly reduced serum testosterone levels relative to mock-treated mice (Fig,. 2), confirming a potentially important physiologic role for LH/EGF receptor cross-talk in testicular

steroidogenesis. Notably, decreased LH pulsatility in humans is associated with lower Leydig cell sensitivity (77, 79) and reduced testosterone production, perhaps due to alterations in the EGFR/MEK kinase pathway.

We present the following model to bring together most of the observations regarding gonadotropin-induced steroidogenesis in Leydig cells (Fig. 2.6). In the short-term (less than 60 minutes), LH induces trans-activation of the EGFR through both extra-cellular (MMP-mediated) and intracellular (cAMP-mediated) means. However, the extra-cellular activation is likely not necessary for steroid production. Rapid EGFR signaling leads to activation of the MAPK cascade, resulting in phosphorylation and mitochondrial translocation of StAR. Finally, increased StAR activity promotes steroidogenesis. With longer LH stimulation (2 hours), LH/EGF receptor cross-talk disappears; however, StAR phosphorylation and translocation to the mitochondria persists. This continued StAR activation may be due to increased StAR production in response to cAMP and PKA signaling, or possibly to other undiscovered pathways. Further studies will be necessary to uncover these potential alternative pathways, as well as to determine the true physiologic importance of LH/EGF receptor crosstalk in testicular androgen production.

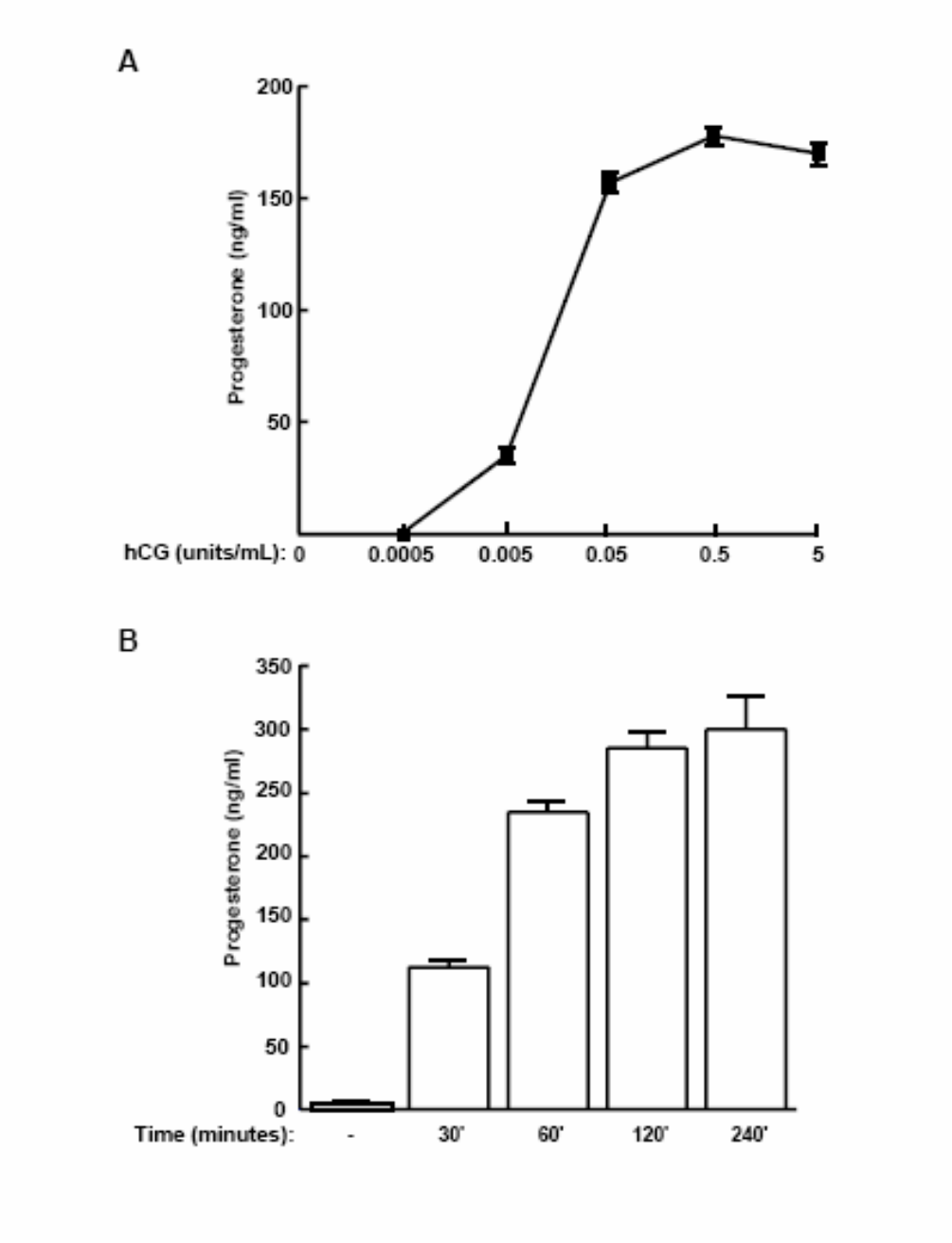

Figure. 2.1: **hCG promotes progesterone production in MLTC-1 Leydig cells in a dose-and time-dependent fashion.** (A) hCG was added to MLTC-1 cells at the indicated concentrations and progesterone levels in the media measured after 30 minutes. (B) 5 units/mL hCG were added to MLTC-1 cells and progesterone levels in the media measured at the indicated times. Each point/bar represents the mean ± S.D. (n=3). All studies were performed at least twice with virtually identical results.

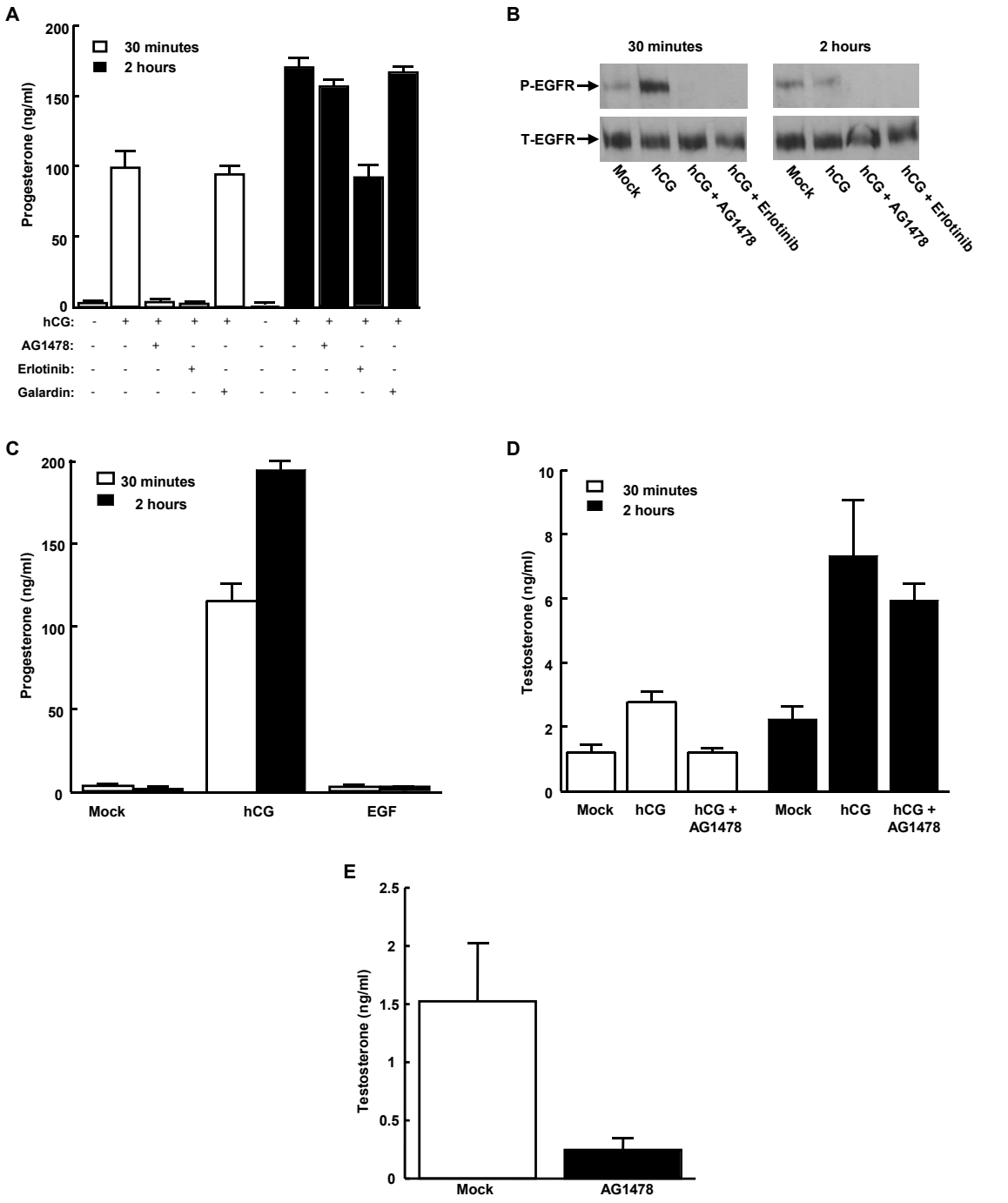

Figure 2.2: **EGFR signaling is important for hCG-induced steroidogenesis in Leydig cells.** (A&B) MLTC-1 mouse Leydig cells were pre-treated with 0.1% ethanol, 20μM AG1478, 20μM Galardin, or 5μM Erlotinb prior to addition of 5 units/mL hCG for 30 minutes or 2 hours. Progesterone content of the media was measured by RIA (A), and cell lysates were examined by Western blot for phosphorylated (B, upper panel), and total (B, lower panel) EGFR. (C) 5 units/mL hCG or 20ng/ml EGF were added to MLTC-1 cells for 30 minutes or 2 hours, and media progesterone content measured by RIA. (D) Primary mouse Leydig cells were isolated from sexually mature male mice, placed in 6-well plates, and serum starved overnight. Cells were then pre-incubated with 0.1% ethanol or 20μM AG1478 for 30 minutes before addition of 5 IU/mL hCG for 30 minutes or 2 hours. Testosterone content in the media was measured by RIA. For all graphs, each bar represents the mean ± SD (n=3). All studies were performed at least three times with nearly identical results. (E) EGF receptor signaling is important for testosterone-production *in vivo*. Seven week old male Mice were

injected with either PBS or PBS containing 20 μM AG1478. Mice were re-injected after 24 hours and serum testosterone levels measured after a total of 36 hours. Each bar represents the mean ± SEM (n=11). Using the student's t-test, p=0.03.

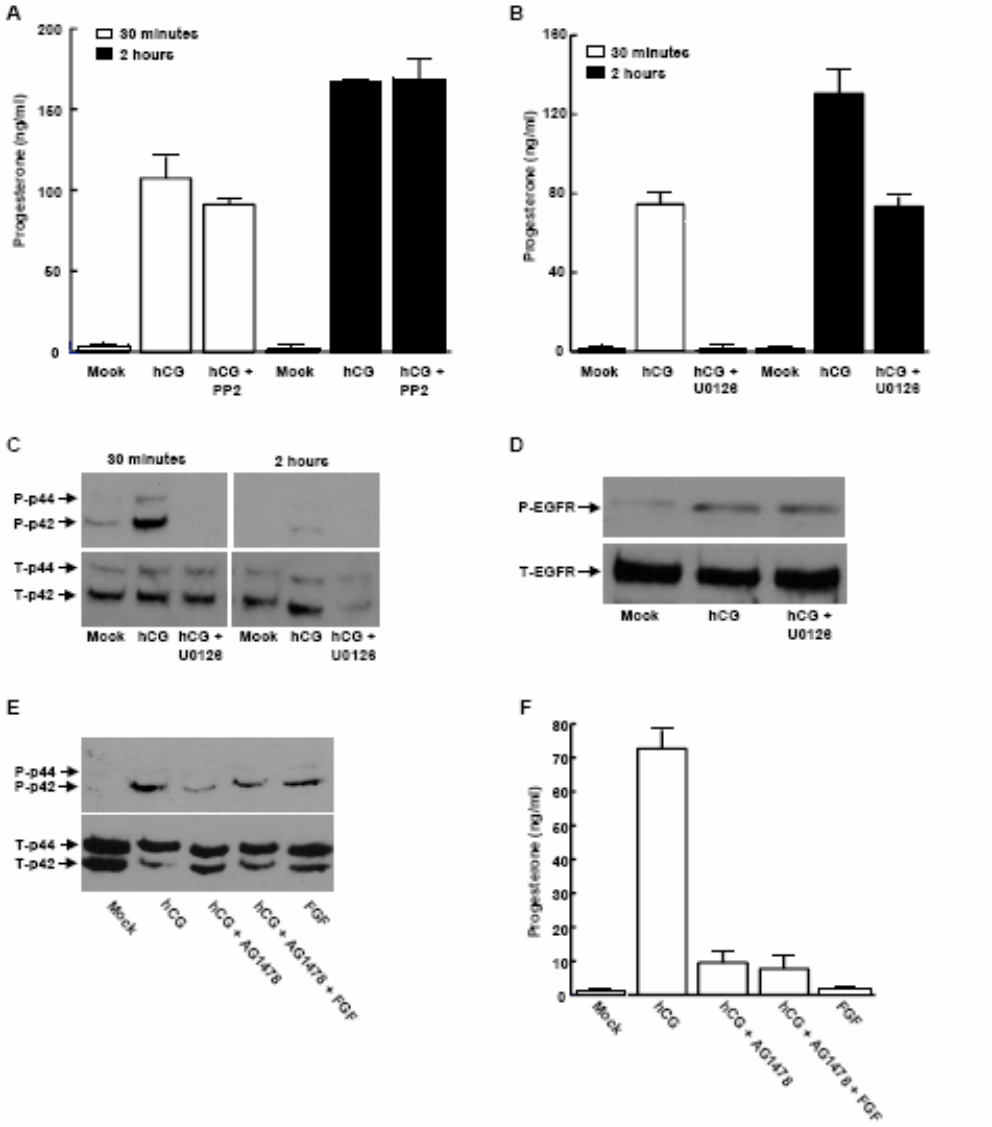

Figure 2.3: **MAPK signaling is necessary for early, but not prolonged, hCG-induced steroidogenesis in Leydig cells.** MLTC-1 cells were pre-incubated with 0.1% ethanol, 20μM PP2 (A, Src inhibitor), or 20μM U0126 (B-D, MEK inhibitor), followed by stimulation with 5 IU/mL hCG for the indicated times. Progesterone content in the media was measured by RIA (A&B). MEK activity in cell lysates was determined by Western blot for phosphorylated p42/p44 (C, upper panel) and total p42/p44 (C, lower panel). (D) EGFR activation occurs upstream of MEK activation. EGFR activation in cell lysates was determined by Western blot for phosphorylated EGFR (D, upper panel) and total EGFR (D, lower panel). (E &F) MEK activation is not inhibited by AG1478 and is not sufficient to promote steroidogenesis. MLTC-1 cells that were pretreated with 0.1% ethanol (mock) or 20 μM AG1478 were incubated with either 5 units/mL hCG, 25 ng/mL FGF-2, or both peptides for 30 minutes. Media progesterone levels were measured by RIA (F), and cell lysates analyzed by Western blot for phosphorylated (upper panel) or total (lower panel) p42/p44. (F). For all graphs, each bar represents the mean ± SD (n=3). All experiments were performed at least twice with essentially identical results.

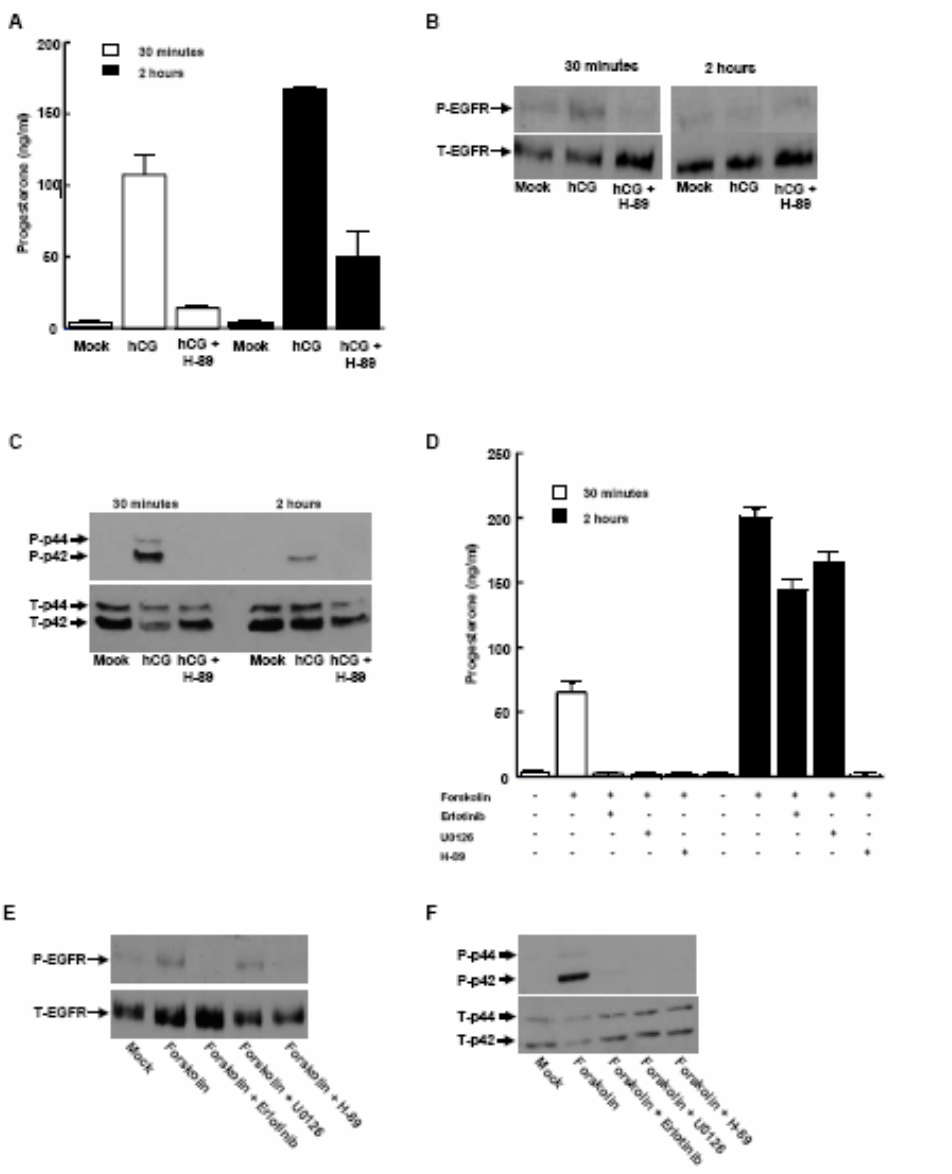

Figure 2.4: **cAMP and PKA signaling are necessary and sufficient for hCG-induced activation of the EGFR and MEK, as well as steroidogenesis, in Leydig cells.** (A-C) MLTC-1 cells were pre-incubated with 0.1% ethanol (mock) or 20μM H-89 (PKA inhibitor), followed by addition of 5 IU/mL hCG for the indicated times. Progesterone content in the medium was measured by RIA (A). Cell lysates were examined by Western blot for phosphorylated and total EGFR (B, upper and lower panels, respectively), and phosphorylated and total p42/p44 (C, upper and lower panels, respectively). (D-F) MLTC-1 cells preincubated for 30 minutes with 0.1% ethanol (mock), 5μM Erlotinib (EGFR inhibitor), 20μM U0126 (MEK inhibitor), or 20μM H-89 (PKA inhibitor), followed by addition of 10μM forskolin for the indicated times. Progesterone content in the media was determined by RIA (D). Cell lystates were examined by Western blot for phosphorylation of the EGFR (E) or p42/p44 (F). For all graphs, each bar represents the mean ± SD (n=3). Each experiment was performed at least three times with nearly identical results.

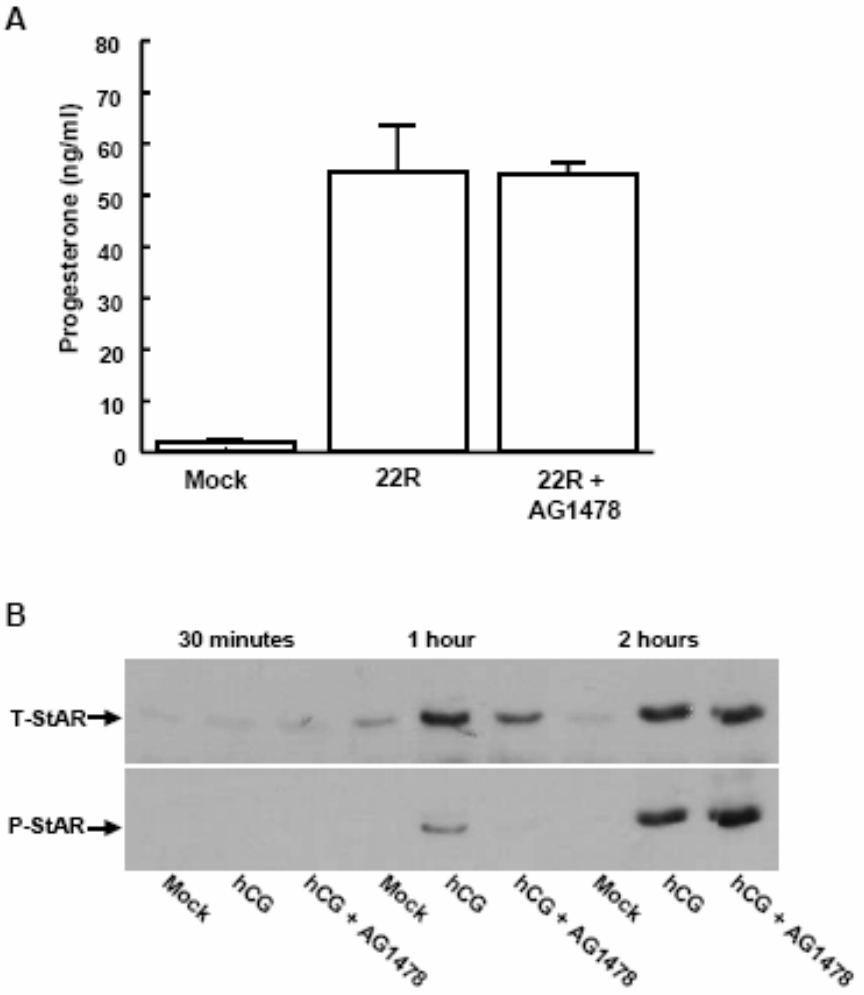

Figure 2.5. **LH receptor-mediated trans-activation of the EGFR regulates early, but not prolonged, phosphorylation and mitochondrial translocation of StAR in Leydig cells.** (A) MLTC-1 cells were pre-incubated for 30 minutes with 0.1% ethanol (mock) or 20μM AG1478 (EGFR inhibitor) followed by addition of 20μM 22R-hydroxycholesterol (Steraloids, Newport, RI) for 30 minutes. Progesterone content in the media was measured by RIA. Each bar represents the mean ± SD (n=3). (B) MLTC-1 cells were stimulated with 5 IU hCG for indicated times after pre-incubation with 0.1% ethanol (mock) or 20μM AG1478. Mitchondria were isolated and examined by Western blot for total StAR (upper) and phosphorylated-StAR. Each experiment was performed at least three times with essentially identical results.

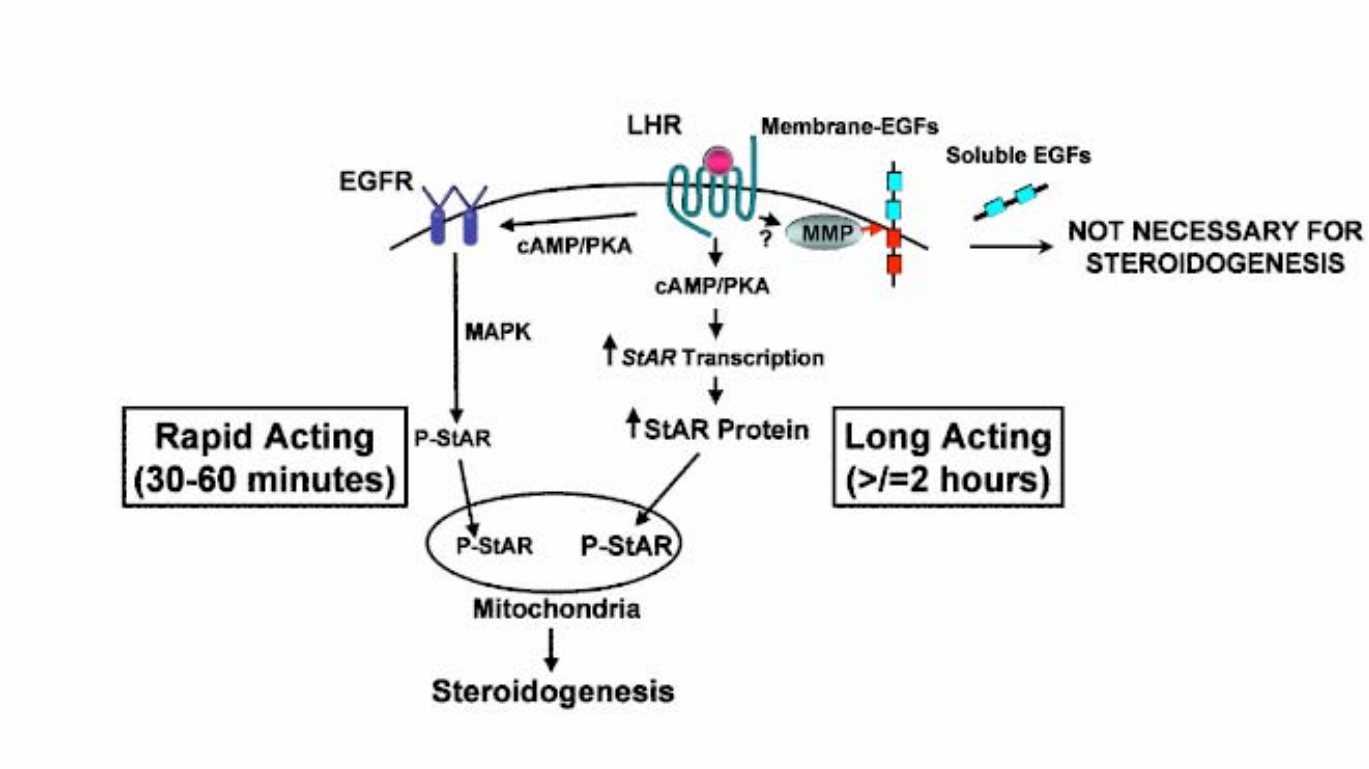

Figure 2.6. **Model for gonadotropin-mediated steroidogenesis in Leydig cells.** Gonadotropin (LH or hCG) activates the LH receptor, leading to a rapid and prolonged increase in cAMP levels and subsequent PKA activity. Early increases in PKA signaling trans-activate the EGFR independent of ligand (left side of figure), leading to activation of the MAPK signaling pathway. By mechanisms that are still not known, MAPK activation leads to phosphorylation of StAR and translocation to the mitochondria, resulting in increased steroidogenesis. At the same time, prolonged PKA signaling leads to increased transcription of StAR mRNA, followed by increased StAR protein expression (middle of figure). By 2 hours, significant MAPK signaling is no longer occurring, nor is it required for StAR phosphorylation and translocation to the mitochondria. Note that LH receptor activation also triggers trans-activation of the EGFR via MMP activation (right side of figure); however, this process is not necessary for steroid production and any time point.

Chapter 3

Testosterone-mediated inhibition of Gβγ-sensitive GIRK activity

Introduction

Steroids regulate the activity of many membrane signaling molecules, including PI3K, Src,and G proteins (80-83). Interestingly, classical steroid receptors localized outside the nucleus, and often associated with the plasma membrane, mediate many of these transcription independent, or nongenomic, steroid-triggered signals.

One well-established and biologically relevant model of nongenomic steroid signaling is *Xenopus laevis* oocyte maturation (29, 84, 85). *Xenopus* oocytes are held in meiotic arrest at prophase I until just prior to ovulation, when gonadotropins stimulate steroid production. Steroids in turn trigger oocytes to progress through the cell cycle until they arrest again at metaphase II of meiosis.

Progesterone has long been considered the physiologic mediator of *Xenopus* oocyte maturation, perhaps signaling via classical *Xenopus* progesterone receptors (PRs) (29, 86, 87). However, evidence suggests that this model is incomplete. First, the classical PR antagonist RU486 does not inhibit progesterone-mediated oocyte maturation (86, 88). Second, elevation or reduction of PR expression in oocytes reveals only small changes in progesterone sensitivity (86, 87). Third, oocytes contain high CYP17 activity that converts progesterone to its equally potent androgen metabolite androstenedione (38, 89, 90); thus, incubating oocytes with progesterone is equivalent to adding two different ligands. Finally, both *in vitro* (91) and *in vivo* (38) measurements of gonadotropin-induced ovarian steroidogenesis reveal extremely low progesterone production but high testosterone secretion, suggesting that the more potent testosterone is the true physiologic mediator of *Xenopus* oocyte maturation. In fact, testosterone

appears to be promoting maturation at least in part through the classical androgen receptor (AR), as reduction of AR expression by RNA interference attenuates androgen-mediated oocyte maturation under conditions of low (approximately 50 nM) testosterone concentrations (30). Interestingly, high testosterone concentrations (500 nM range) still trigger maturation in oocytes with reduced AR expression (30), suggesting that additional receptors are utilized to promote meiotic progression when steroid concentrations are elevated.

How do androgens and progesterone trigger *Xenopus* oocyte maturation? The current "Release of Inhibition" model proposes that constitutive G protein signaling in resting oocytes maintains meiotic arrest. Addition of steroids attenuates this inhibitory signal, allowing meiotic progression (34, 41, 85). While both Gαs and Gβγ contribute to maintaining meiotic arrest (33, 34, 41, 85, 92), Gβγ appears to be essential for this process. Over-expression or sequestration of Gβγ inhibits or enhances steroid-triggered oocyte maturation, respectively (35, 41), and stimulation of the Gβγ-coupled muscarinic 2 receptor (M2R) inhibits steroidmediated maturation (30).

Although changes in Gβγ expression alter steroid-mediated maturation (35, 41), steroids themselves have not been shown to directly modify Gβγ-mediated signaling. To address this question and to test the release of inhibition model, we studied the effects of steroids on G protein regulated Inward Rectifying Potassium channels (GIRKs). GIRK1/GIRK2 heterodimers are extremely sensitive to changes in Gβγ activity, with elevated Gβγ signaling promoting increased GIRK activity, or potassium flux, at the plasma membrane (93-96) (Fig.3.1). These channels can therefore be expressed in *Xenopus laevis* oocytes and used as markers of Gβγ signaling.

Using GIRKs, we confirmed that constitutive Gβγ activity occurred in resting *Xenopus* oocytes, and that testosterone and progesterone rapidly reduced this baseline activity. Similar to earlier maturation studies, reduction of AR expression abrogated androgen mediated inhibition of GIRK activity at low, but not high, agonist concentrations. Interestingly, androgen bound to the *Xenopus* AR at low concentrations, but bound to both the *Xenopus* AR and PR at high

concentrations. In contrast, progesterone bound to both the *Xenopus* AR and PR with equally high affinities. Together, these results suggest that cross-talk may occur between steroids and their receptors during nongenomic steroid-triggered processes.

Materials and Methods

Molecular Biology

The cDNAs encoding rat GIRK1, mouse GIRK2 (both generous gifts from L. Jan, University of California at San Francisco) and HA-tagged Muscarinic 2 receptor (M2R, purchased from Guthrie/UMC cDNA resources) were all cloned into the vector pGEMHE (gift from L. Jan, University of California at San Francisco). The cRNA encoding the Renal Outer Medullary potassium channel (ROMK) was a generous gift from Dr. Chou-Long Huang (University of Texas Southwestern Medical Center). *In vitro* transcription was performed as previously described in (41) to make cRNA for subsequent injection.

Oocyte Preparation and RNA injection

Stage V-VI oocytes were isolated from unprimed female *Xenopus Laevis* (Nasco, Fort Atkinson, WI) and treated as previously described using collagenase A (97, 98). For every preparation, dose response curves with testosterone were performed to determine their steroid sensitivities (41).

Equal concentrations of the cRNAs encoding GIRK1 and GIRK2 were mixed, and approximately 25ng of total cRNA was injected into each oocyte. For the studies using M2R, approximately 10ng of cRNA encoding the HA-tagged M2R was injected into each oocyte.

For the AR RNA interference studies, cRNAs were generated that encoded the forward and reverse AR sequences. Equal amounts of the two RNAs were mixed together and heated to 90 °C, followed by gradual cooling to room temperature. Then, 15 ng of the double-stranded RNA was injected into oocytes, followed one hour later by injection of cRNAs encoding GIRKs 1 and 2. Assays were then performed 35-48 hours later. PR expression was lowered by RNA interference using the identical strategy with forward and reverse PR cRNAs.

Two-electrode voltage-clamp recording

Oocytes were injected with cRNA for GIRK1/GIRK2 and/or M2R as indicated above. Two electron voltage-clamp readings were taken 2 days after cRNA injections**.** Oocytes were bathed at 23°C in a solution of 96 mM KCl, 1 mM MgCl2, and 5 mM HEPES (pH 7.5 by KOH). Current-voltage relationships (–100 to +100 mV, in 25-mV steps) were measured in oocytes as described (99) by two-electrode voltage clamp using an OC-725C oocyte clamp amplifier (Warner Instruments, Hamden, CT), pCLAMP7 software (Molecular Devices, Sunnyvale, CA), and a Digidata 1200A digitizer (Molecular Devices). The resistance of current and voltage microelectrodes (filled with a 3 M KCl solution) was 1–2 M .

After the initial readings, oocytes were treated with steroid or ethanol at the indicated concentrations for the indicated times, at which point the measurements were repeated. Ethanol concentrations were kept the same at 0.01%. For the experiments using oocytes expressing M2R, 30 μM carbachol (Sigma, St. Louis, MO) was added to oocytes followed shortly by the addition of testosterone.

M2R expression

HA-tagged M2R expression was confirmed by Western blot using an anti-HA antibody (41). Oocytes were broken apart by pipetting with 20 μl/oocyte of lysis buffer (150mM NaCl, 2mM EDTA, 0.5mM sodium vanadate, 2mM NaF, 1% Tx-100, 20mM Tris, pH7.6) containing 100μg/ml PMSF, 2μg/ml Aprotinin, 0.5 μg/ml Leupeptin and 1μg/ml Pepstatin (Sigma) at 4ºC. Lysates were microcentrifuged at full speed for 10 minutes, and the supernatant was removed and diluted 1:2 in 2X Laemmli sample buffer with 10% β-mercaptoethanol (Sigma-Aldrich)**.** Samples were separated on 10% polyacrylamide gels and transferred to Immobilon membranes (Millipore, Billerica, MA). Membranes were blocked with 5% milk in TBST (100mM NaCl, 0.1% Tween-20, 50mM Tris, pH7.4) for 1 h, incubated overnight at 4ºC with (1:4000) rabbit anti-HA (Covance, Princeton, NJ), washed three times with TBST, incubated 1 hr at room temperature with (1:4000) horseradish peroxidase-conjugated goat anti-rabbit antibody, and washed another

three times with TBST. Blots were then treated with ECL-Plus (GE Healthcare, Piscataway, NJ) to visualize the proteins.

Steroid Binding Studies

Steroid binding studies were performed in COS cells as described previously (30). Progesterone and testosterone affinities were determined by direct binding assays using [1, 2, 6, 7–3H(N)]-testosterone or [1, 2, 6, 7–3H(N)]-progesterone (Perkin-Elmer, Boston, MA). RU5020, RU486, and R1881 binding affinities were determined by competition assay using 1 nM radiolabeled testosterone or progesterone with increasing concentrations of unlabeled test steroid. Kds were then determined using the Prism software (Graphpad Software, Inc, San Diego, CA).

Oocyte Maturation Assays

Steroid-mediated maturation assays were performed as described (38). For the inhibition assays, 1 μM RU5020 and/or 1 μM R1881 was added 30 minutes prior to the addition of the indicated concentrations of progesterone, and germinal vesicle breakdown recorded 16 hours later.

Transcription Assays

Transcription assays were performed as previously described (30, 38). Briefly, COS and CV1 cells were grown in complete medium consisting of DMEM (Fisher), 10% bovine serum (Invitrogen), 100 IU/ml penicillin, and 0.1 mg/ml streptomycin (Invitrogen).

For the PR transcription assays, cells were transfected in 12-well plates using lipofectamine (Invitrogen). Cells were transfected with 0.8 ug *Xenopus* PR (87) in mammalian expression vector pcDNA3.1, 1.2 ug mouse mammary tumor virus-luciferase plasmid, and 5 ng of cytomegalovirus-β-galactosidase plasmid. Cells were incubated with the mixture for 4 hours and placed in complete medium with 5% charcoal-filtered serum and the indicated steroids. After

24 hrs cells were incubated with the indicated steroid concentration and lysed after 16hrs. Extracts were analyzed by using the Promega luciferase assay system and Perkin-Elmer Galactostar kit.

For the AR transcription assays, CV1 cells were transfected by calcium phosphate precipitation with the MMTV-luciferase plasmid and pcDNA3.1 containing the XeAR cDNA coding sequence (38). Cells were then incubated in CM containing 5% charcoal-stripped fetal bovine serum and the indicated steroids for 48 hours, and luciferase and β-galactosidase expression measured as above.

Results

Testosterone inhibits constitutive GIRK channel current in Xenopus laevis oocytes

GIRKs 1 and 2 were over-expressed in *Xenopus* oocytes by cRNA injection. Oocytes were then placed in the high potassium solution and a two-electron voltage clamp chamber was used to measure the potassium current. Representative graphs from currents measured in individual oocytes are shown in Figure 3.2. Basal inward potassium current was detected in unstimulated oocytes (Fig. 3.2A and 3.2C), confirming that resting *Xenopus leavis* oocytes contained constitutive Gβγ signaling. Notably, the general potassium channel blocker barium chloride completely inhibited this inward signal, verifying that the detected current was indeed due to potassium flux (data not shown). Addition of 100 nM testosterone led to a 30% decrease in potassium current at 5 minutes (Fig. 3.2D versus 3.2C), while addition of the carrier 0.01% ethanol reduced the potassium current by only 5% (Fig. 3.2B versus 3.2A).

To characterize the time course of testosterone's actions on GIRK activity, oocytes expressing GIRK1/2 were treated with either ethanol or testosterone, and currents were sequentially measured for up to 10 minutes. The average readings in three different oocytes from the same batch are shown in Fig. 3.3A. Ethanol mildly suppressed GIRK activity in a linear time-dependent fashion throughout the 10 minutes, reaching a maximum of approximately 10% inhibition. In contrast, testosterone suppressed GIRK activity in a logarithmic pattern, with the greatest change in inhibition occurring by 5 minutes (30%). This very rapid effect of testosterone on GIRK activity supports the concept that testosterone is signaling in a nongenomic fashion in *Xenopus* oocytes.

Testosterone effects on GIRK activity are mediated by Gβγ

The premise of these studies is that all GIRK activity is Gβγ dependent; thus, any effects on GIRK-mediated potassium influx should be directly mediated by Gβγ. The specific sensitivity of GIRKS to Gβγ is well-established (93). However, to confirm that testosterone was not simply

suppressing all potassium currents in a non-specific fashion, the constitutive potassium channel ROMK was over-expressed in *Xenopus* oocytes, and potassium currents measured in the presence of ethanol or testosterone. While testosterone inhibited potassium influx in oocytes from the same batch that were expressing GIRK1/2, the steroid had no effect on potassium influx in oocytes expressing ROMK (Fig. 3.3B). This result confirmed that the androgen effects were specific to GIRK channels, and that Gβγ signaling was therefore being altered in response to testosterone.

If testosterone is indeed altering Gβγ signaling, then over-stimulation of endogenous Gβγ should further increase GIRK activity and block the suppressive effects of testosterone. To test this hypothesis, the Gβγ-coupled muscarinic 2 receptor (M2R) was co-expressed with GIRK1/2 in oocytes and potassium currents measured. M2R expression was confirmed by Western blot (Fig. 3.3D). As expected, testosterone suppressed baseline GIRK activity by approximately 30% at five minutes. Similarly, as anticipated, the M2R agonist carbachol increased GIRK activity by approximately 30% at two minutes. Interestingly, subsequent addition of testosterone to the carbachol-treated oocytes did not suppress GIRK activity at five minutes, suggesting that the potent stimulatory effect of carbachol on Gβγ signaling through the M2R was preventing the suppressive effects of testosterone (Fig. 3.3C). The ability of M2R signaling to block androgen effects on GIRK activity mirrors previous studies where stimulation of the M2R blocked steroid-mediated maturation (30), supporting the hypothesis that interplay occurs between steroids, maturation, and Gβγ signaling in *Xenopus* oocytes.

The pharmacology of androgen-mediated suppression of Gβγ signaling and maturation are similar

If testosterone's actions are mediated by a specific receptor, then the steroid's effects on GIRK activity should be dose-dependent and saturable. Accordingly, testosterone inhibited GIRK activity in a dose-dependent fashion, reaching a maximum inhibition of 30% at 100 nM, with an EC50 around 10 nM (Fig. 3.4A). Notably, testosterone concentrations higher than 100 nM had no

greater inhibitory effects on GIRK activity (data not shown). Interestingly, the dose-response for testosterone-mediated inhibition of GIRK activity was nearly identical to that of testosterone-triggered maturation of oocytes from the same batch on the same day, suggesting that testosterone may be regulating both processes via the same receptor (Fig. 3.4A, bottom).

As another pharmacologic comparison of androgen effects on maturation and GIRK signaling, we tested the consequences of the compound R1881 on GIRK currents. Interestingly, R1881 binds tightly to the *Xenopus* AR and is a potent promoter of *Xenopus* AR-mediated transcription; however, it is unable to promote *Xenopus* oocyte maturation, even at high concentrations (30, 100). Thus, R1881 is thought to be a selective androgen receptor modulator (SARM) that specifically activates genomic but not nongenomic AR mediated signaling. Accordingly, R881 had no effect on GIRK activity in *Xenopus* oocytes when compared with testosterone (Fig. 3.4B).

The classical AR is necessary for testosterone-mediated suppression Gβγ signaling when steroid concentrations are low.

Reduction of AR expression by RNA interference abrogates MAPK activation and oocyte maturation triggered by low concentrations of testosterone, suggesting that the classical AR mediates nongenomic signaling under these conditions (30, 38, 100). To determine whether the classical AR similarly regulates testosterone-mediated inhibition of GIRK activity, potassium currents were measured in oocytes where AR expression was reduced by RNA interference (30, 47). In oocytes injected with double-stranded AR cRNA, AR levels were lowered by approximately 60-70%, while expression of MNAR, a nonspecific protein also involved in regulating Gβγ signaling (47), was unaffected (Fig. 3.5A). In accordance with maturation studies (30), low concentrations of testosterone (50 nM) were no longer sufficient to inhibit GIRK activity in oocytes with reduced AR expression when compared with mock injected oocytes, while high concentrations of testosterone (500 nM) still suppressed GIRK activity (Fig. 3.5B).

These results confirm that the classical AR is required to regulate nongenomic Gβγ signaling at low androgen concentrations, while additional receptors may also be involved in mediating nongenomic signaling when androgen levels are high.

Progestins and Androgens can bind to both the Xenopus AR and PR

What other receptor might testosterone be utilizing at high concentrations? One possibility is that testosterone binds to and activates the classical PR under these conditions. In fact, the androgens testosterone, androstenedione, and R1881 were all capable of binding to the *Xenopus* PR, although with Kds that were 10-100 fold higher than those for binding to the *Xenopus* AR (Table 3.1). Importantly, the Kd for testosterone binding to the AR was 0.8 nM, but was 170 nM for binding to the PR. These values may explain why the AR was required for 50 nM, but not 500 nM, testosterone to suppress Gβγ signaling and trigger oocyte maturation. Perhaps at higher concentrations, testosterone can bind to and signal via the *Xenopus* PR. As proof in principle that androgens can activate a signal via the *Xenopus* PR, the androgens testosterone and androstenedione were shown to indeed activate PR mediated transcription, albeit at lower levels relative to progesterone (Fig. 3.6A).

In contrast to androgen/PR interactions, progesterone bound to both the *Xenopus* AR and PR with equally high affinities (Kds approximately 1 nM). Furthermore, progesterone significantly increased AR-mediated transcription (over 5-fold induction) (Fig. 3.6B). Since progesterone binds equally well to both the Xenopus PR and AR, and promotes AR-mediated transcription, progesterone may likewise mediate nongenomic AR-mediated signals. Notably, similar to testosterone, progesterone rapidly attenuated Gβγ-mediated GIRK activity in oocytes (Fig. 3.5C), perhaps via signaling through both the PR and AR.

RU486 binds poorly to the Xenopus PR.

As mentioned, one of the conundrums of progesterone-mediated oocyte maturation is that the classical PR antagonist RU486 does not block progesterone-triggered maturation. This ineffectiveness of RU486 appears to be due to its extremely low affinity for the *Xenopus* PR (Table 3.1). Interestingly, the *Xenopus* PR contains a cysteine rather than glycine at residue 376 (Fig. 3.7A), and this cysteine substitution is known to decrease RU486 binding in the chicken and hamster PRs (82, 101). In contrast, the *Xenopus* AR contains a glycine at the equivalent position (579), likely explaining why RU486 bound with high affinity to the AR (Table 3.1). As would be expected, when this glycine in the AR is mutated to cysteine, the receptor's affinity for RU486 is markedly reduced (data not shown).

RU5020 is a Selective Progesterone Receptor Modulator (SPRM)

Like progesterone, the progestin RU5020 also bound to the AR, although with 10-fold lower affinity relative to its binding to the PR. Importantly, despite its high affinity for the *Xenopus* PR, RU5020 does not promote oocyte maturation, but still activates *Xenopus* PRmediated transcription (86). Thus, similar to the effect seen with R1881 on the *Xenopus* AR, RU5020 appears to be a SPRM that specifically activates genomic but not nongenomic PR mediated signaling.

Blockade of both the AR and PR significantly reduces progesterone-mediated oocyte maturation.

As mentioned, a big problem studying progesterone-mediated oocyte maturation *in vitro* is that traditional PR antagonists have minimal inhibitory effects. Our binding studies may explain this problem, as no one ligand sufficiently blocks both the AR and the PR. To address this issue, we attempted to block progesterone-mediated maturation using the high affinity AR ligand R1881 and the high affinity PR ligand RU5020 as competitive antagonists. Addition of R1881 and RU5020 together only promoted a small amount of oocyte maturation (Fig. 3.7B), indicating

slight partial agonistic activity. R1881 slightly inhibited progesterone mediated maturation, most likely due to specific blockade of progesterone and its metabolite, androstenedione, binding to the *Xenopus* AR. RU5020 inhibited progesterone-mediated maturation slightly more than R1881, likely because of its ability to bind tightly to the PR but still moderately to the AR. Finally, the combination of R1881 and RU5020 inhibited progesterone-mediated maturation by 50-60%, probably the result of significant blockade of both classical receptors (Fig. 3.7B). Notably, maturation could not be reduced to zero by the inhibitors, most likely due to the partial agonist qualities of R1881 and RU5020, as well as the extended incubation times used for a maturation assay (16hours).

Discussion

In these studies, we have demonstrated a simple, reproducible method to directly measure rapid, nongenomic effect of steroids on Gβγ signaling in real-time using intact cells. This system involves measuring currents in oocytes over-expressing the G protein-coupled inward-rectifying potassium channels GIRK1 and GIRK2, which have previously been characterized to be very specific and sensitive to Gβγ-mediated signaling (95, 96, 102). We have then used this system to test and confirm the validity of the release of inhibition hypothesis regarding steroid-mediated maturation of *Xenopus* oocytes.

First, we confirmed that resting oocytes contained constitutive GIRK activity. Earlier studies showed that this constitutive GIRK activity could be reduced by over-expression of proteins that sequester Gβγ and enhanced by over-expression of Gβγ (103). Similarly we verified here that stimulation of the over-expressed Gβγ-linked G-protein coupled receptor M2R enhanced constitutive GIRK activity (Fig. 3.3). Together, these studies corroborate that GIRK activity is regulated tightly by Gβγ signaling in *Xenopus* oocytes, and that measurement of potassium current through GIRK1/2 is a sensitive and specific indicator of Gβγ signaling in our hands.

Second, we demonstrated that testosterone, the physiologic mediator of *Xenopus* oocyte maturation, attenuated resting GIRK activity in a dose dependent fashion (Fig. 3.3). Since, as mentioned, GIRK activity is mediated almost exclusively by Gβγ, these androgen-induced changes are very likely to be due to specific alterations in Gβγ-mediated signaling. In support of this interpretation, increasing Gβγ signaling by stimulating the M2R with carbachol blocked the inhibitory effects of testosterone on GIRK activity (Fig. 3.3). Furthermore, testosterone had no effect on ROMK-mediated potassium currents, ruling out the possibility that the steroid was nonspecifically suppressing potassium influx (Fig. 3.3B).

Notably, each batch of *Xenopus* oocytes can have significantly different dose-response curves for testosterone-induced maturation. We found that the dose responses for testosterone-mediated GIRK inhibition and maturation matched nearly perfectly for every preparation tested

(Fig. 3.4A and data not shown), suggesting that both testosterone-regulated events are part of the same process, and are being mediated by the same receptor.

What, then, are the receptors that regulate testosterone- and progesterone-mediated inhibition of Gβγ signaling and eventual oocyte maturation? Based on previous work and on our functional and binding data, we propose the following model depicted in figure 7C. *In vivo*, androgens (mainly testosterone) are the primary regulators of oocyte maturation (38). In fact, inhibition of androgen production markedly reduces and delays gonadotropin-mediated oocyte maturation and ovulation, respectively (100). Testosterone mediates maturation primarily via its high affinity interactions with the AR (thick line); however, when androgen levels are high enough, androgens may promote maturation via binding to the PR (thin dotted line). In contrast, addition of progesterone to oocytes *in vitro* is far more complicated. First, progesterone is partly metabolized to androstenedione, which binds to the XeAR at low to moderate concentrations, but can cross-react with the PR at high levels. In addition, progesterone itself binds with equally high affinity to both the *Xenopus* PR and AR, and even promotes significant transcription mediated by either receptor. Progesterone may therefore promote maturation via either receptor, regardless of whether it is metabolized to androstenedione.

This proposed model is compelling, as it reconciles our current data with the previous work described in the introduction. 1) Our data explain why RU486 does not block progesterone-mediated maturation by demonstrating that RU486 binds to the *Xenopus* PR binds with extremely low affinity, likely due to a cysteine to glycine substitution in position 376 of the ligand binding domain. In contrast, RU486 binds with high affinity to the *Xenopus* AR, which contains the required glycine at the equivalent position 579. The ability of RU486 to bind with high affinity to the AR likely explains why it is actually a weak agonist of oocyte maturation (88) when added *in vitro*. 2 & 3) Our data explain why reduction or overexpression of the *Xenopus* PR in oocytes has real but only small effects on progesterone-mediated maturation. In addition to CYP17-mediated conversion of progesterone to the androgen androstenedione, progesterone itself binds with high

affinity to the *Xenopus* AR. Thus, reduction of PR expression lowers PR-, but spares AR-mediated maturation, resulting in only a partial reduction of progesterone-mediated signaling and maturation. 4) Our data confirm and explain why reduction of AR expression by RNA interference blocks androgen-mediated effects on Gβγ, MAPK signaling, and maturation only at steroid concentrations in the 50 nM range. At higher concentrations, androgens can bind to, and likely activate, the PR to regulate nongenomic signaling and maturation in oocytes.

Notably, RU5020 does not promote nongenomic steroid-triggered oocyte maturation, nor does it activate any other transcription-independent pathways in oocytes. However, RU5020 is known to activate nongenomic human PR-induced Src and MAPK signaling in somatic cells (104). One difference could be that XePR lacks the important PXXP motif that is found in the human PR and in fact is necessary for Src activation. In addition, Src is not activated by steroid in oocytes, and does not appear to be important for steroid-triggered maturation (47). These differences highlight that classical steroid receptors can trigger multiple nongenomic signals, depending upon both the cell type and the receptor sequence/structure. While Src activation may predominate in somatic cells, regulation of G protein signaling is most important in oocytes. In addition, classical steroid receptors can use multiple means of activating G proteins, including direct interactions (105), indirect interactions via the scaffold molecule striatin (106), or, as may be the case in *Xenopus* oocytes, indirectly via the modulator of nongenomic actions of steroid receptors (MNAR) protein (47).

As one final note, progesterone-mediated inhibition of Gβγ signaling could still be due to activation via a nonclassical steroid receptor, such as the recently described mPR (39). This possibility seems unlikely, however, as the progestin RU5020 does not bind to mPR (107), yet it partially blocks progesterone-mediated maturation in *Xenopus* oocytes. Furthermore, as mentioned, manipulation of *Xenopus* PR levels does in fact result in small but reproducible changes in progesterone-mediated maturation (86, 87), suggesting that this classical receptor plays at least a partial role in regulating nongenomic progesterone signaling. Finally, the evidence

from RNA interference and pharmacology studies are relatively strong that the classical AR regulates androgen-mediated maturation at 10-100 nM concentrations of ligand; therefore, the likelihood that progesterone uses a completely different family of receptors to regulate oocyte maturation seems low. Notably, to date, no mPR isoforms have been shown to bind to any androgen (39, 107).

In sum, these studies introduce a novel method for detecting rapid nongenomic steroid effects on G protein signaling at the membrane. Furthermore, these data demonstrate that considerable cross-talk may occur between steroids and their receptors during *Xenopus* oocyte maturation, underscoring the importance of redefining the novel pharmacology of nongenomic versus genomic steroid signaling.

	XeAR	XePR
Progesterone (nM)	1.2 ± 0.6	1.9 ±0.4
RU5020 (nM)	51 + 3.5	1.6 ± 0.1
RU486 (nM)	4.4 ± 2.2	1880 ± 594
Testosterone (nM)	0.8 ± 0.1*	170 ± 16
Androstenedione (nM)	44 ± 24*	450 ± 212
R1881 (nM)	0.7 ± 0.1*	309 ± 18

Table 3.1: Binding Properties (Kd) of the Xenopus AR and PR. COS cells were transiently transfected with either the XeAR or XePR. All values represent the mean + S.D. for three experiments except for RU5020 binding to the XeAR, which was performed twice. The values for progesterone and testosterone represent direct binding with radiolabeled steroid, while the rest were derived from competition studies using the indicated unlabeled steroid competing with 1 nM radiolabeled progesterone (for PR) or testosterone (for AR). *values from reference 15 and shown here to complete the comparisons. (Binding assay performed by Michelle Jamnongjit Ph.D.)

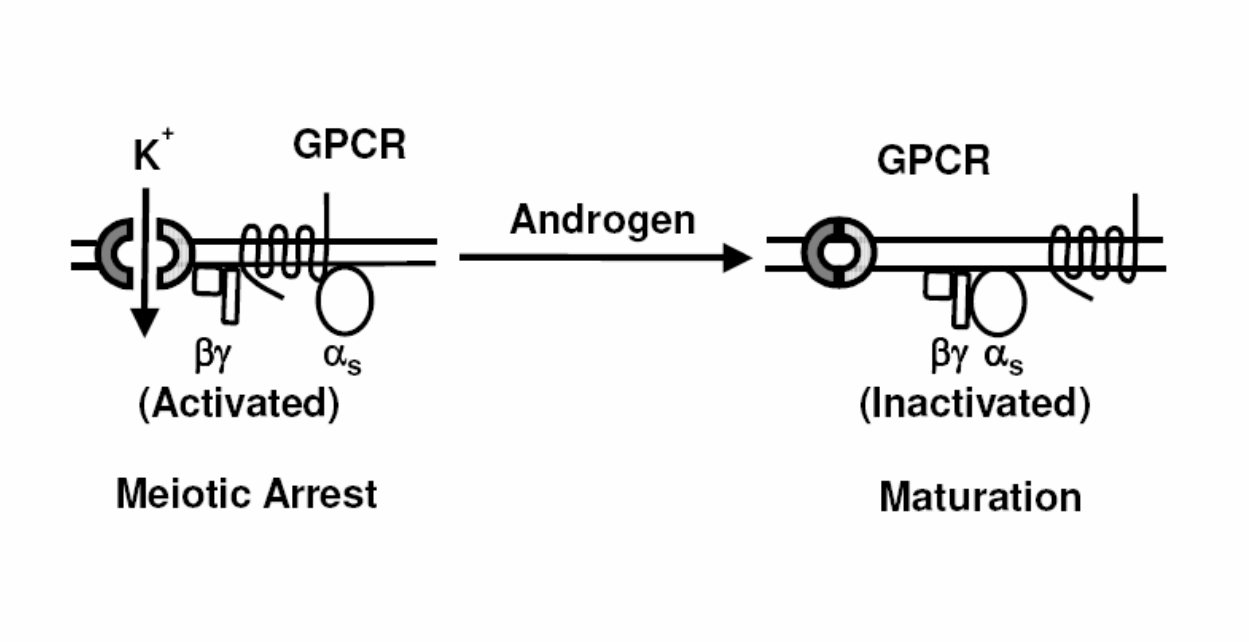

Figure 3.1: Proposed model of androgen-mediated inhibition of Gβγ-sensitive GIRK activity. *Xenopus laevis* oocytes contain constitutive Gβγ signaling, perhaps via a constitutively activated G protein-coupled receptor (GPCR), that can be detected by over-expressing exogenous GIRK heterodimer (in our case, GIRK1 and GIRK2). This results in a detectable inward potassium flux under conditions of high extracellular potassium (left). Through uncharacterized mechanisms that involve the classical AR and the scaffold molecule MNAR (not shown here), addition of androgen attenuates the constitutive Gβγ signaling, resulting in closure of the GIRK channel and reduced inward potassium current (right).

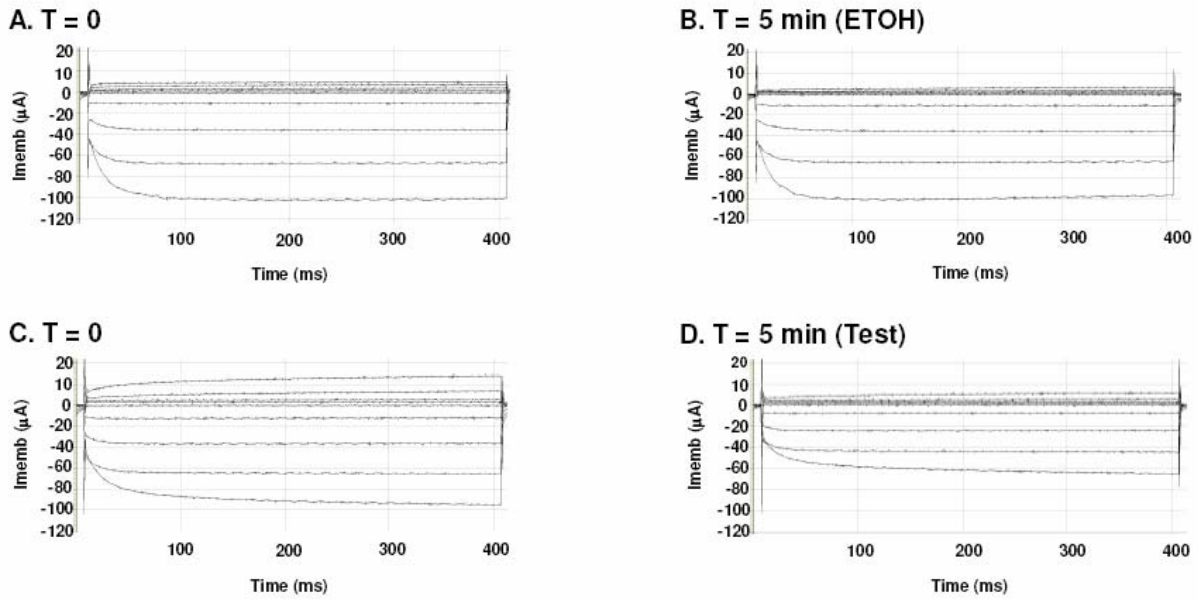

Figure 3.2. Testosterone reduces endogenous GIRK activity in Xenopus laevis oocytes. Injection of cRNAs encoding GIRK1 and GIRK2 led to constitutive inward potassium currents under conditions of high potassium concentration, as measured using a two electrode voltage clamp (TEVC). A) Current was measured in an individual oocyte prior to ethanol (ETOH) addition. B) Current was measured in the same oocyte as (A) five minutes after addition of 0.01% ethanol. C) Current was measured in an individual oocyte prior to testosterone (Test) addition. D) Current was measured in the same oocyte as (C) five minutes after addition of 100 nM testosterone. Currents are indicated as µA on the axis, with the time indicated in milliseconds on the x-axis. Each individual curve represents the current measured at a given voltage from –100 to +100 mV in 25-mV steps. These studies were repeated in more than 100 oocytes with nearly identical results.

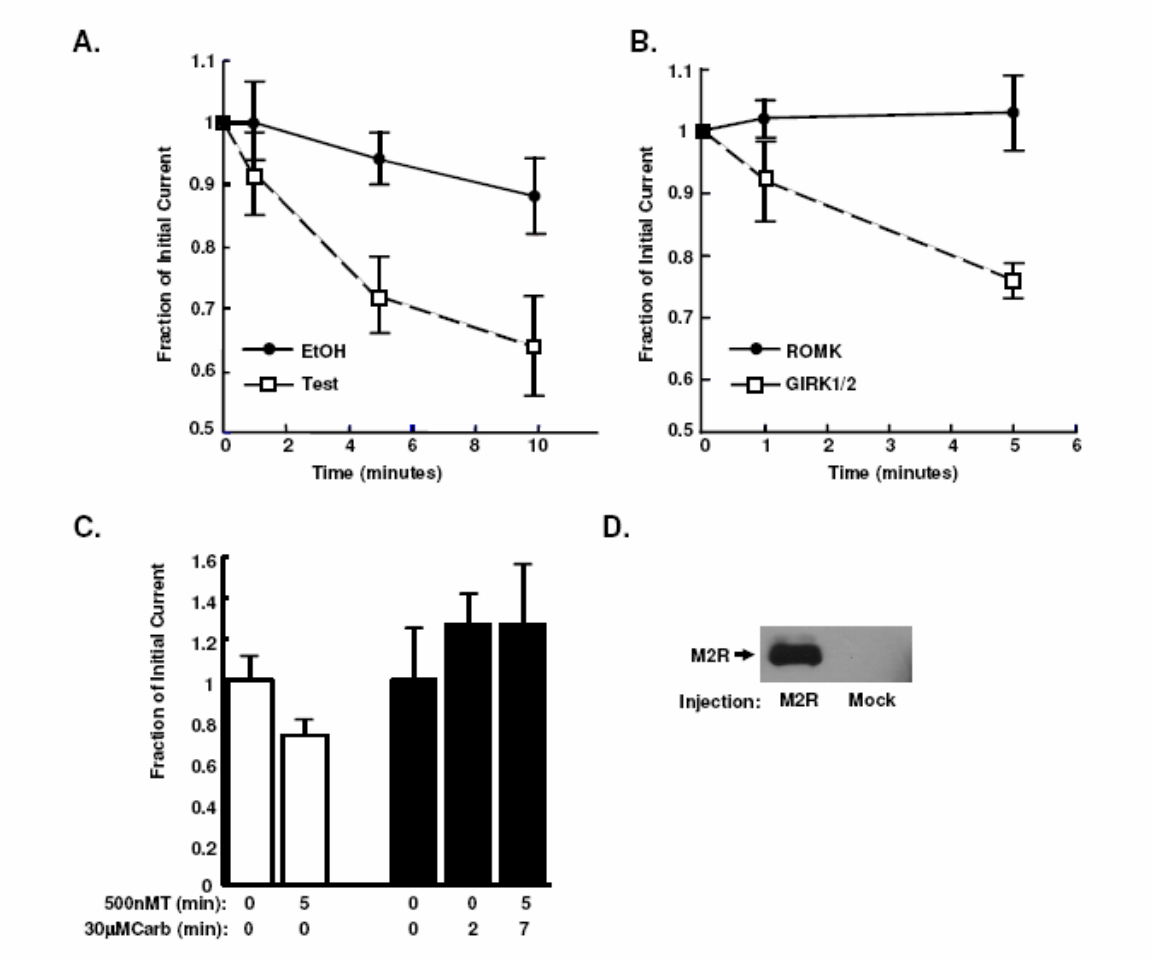

Figure 3.3: Rapid testosterone-mediated inhibition of GIRK activity is mediated through Gβγ. A) Testosterone-mediated inhibition of GIRK signaling is dose-dependent. 100nM testosterone (open squares) rapidly inhibited GIRK activity in a logarithmic pattern, reaching a nadir after approximately 5 - 10 minutes. Ethanol-mediated inhibition was slower and linear (filled circles). The y-axis represents the fraction of the original maximum current. Each point represents the mean +/- S.D. (n=3). B) Testosterone (100 nM) did not inhibit potassium flux mediated by ROMK (filled circles), but still blocked constitutive Gβγ-regulated GIRK activity in oocytes from the same preparation (open squares). C) Activation of endogenous Gβγ blocks testosterone-mediated inhibition of constitutive GIRK activity. Oocytes were injected with cRNAs encoding the muscarinic 2 receptor (M2R), GIRK1, and GIRK2. Addition of 500 nM testosterone reduced constitutive GIRK activity (open bars) by 30% at five minutes, while addition of the M2R agonist carbachol (30μM) increased GIRK activity by approximately 30% at two minutes (first two filled bars). Subsequent addition of 500 nM testosterone to the carbachol-treated oocytes no longer attenuated GIRK activity (last filled bar). Each bar represents the means +/- S.D. (n=3), and this experiments was performed twice with similar results. D) Injection of cRNA encoding the HA-tagged M2R into oocytes resulted in detectable M2R protein by Western blot.

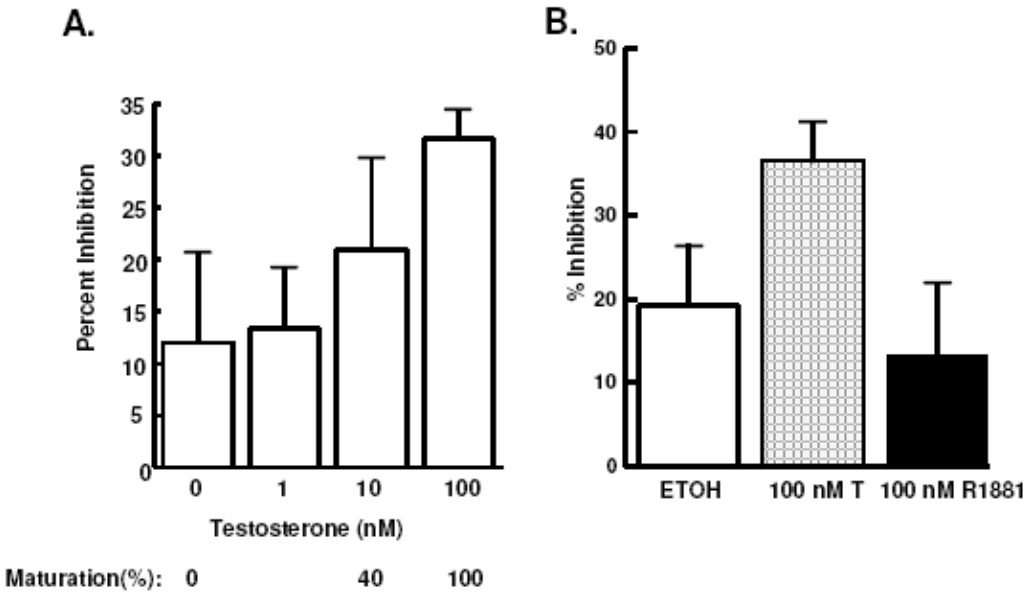

Figure 3.4: Testosterone-mediated inhibition of GIRK activity was dose dependent and correlated with androgen-triggered maturation. Oocytes expressing GIRK1/2 were treated for 10 minutes with testosterone at the indicated concentrations. The percent inhibition of GIRK activity is shown on the y-axis, with the testosterone concentrations shown on the x-axis. The percent maturation of oocytes from the same batch on the same day are shown below the graph. Each bar represents the mean +/-S.D. (n=3). B) The SARM R1881 did not attenuate GIRK activity. Oocytes expressing GIRK1/2 were treated with either ethanol (ETOH) or 100 nM testosterone or R1881 and potassium flux measured at 5 minutes. Each bar represents the mean +/- S.D. (n=3). All experiments for this figure were performed at least three times with similar results.

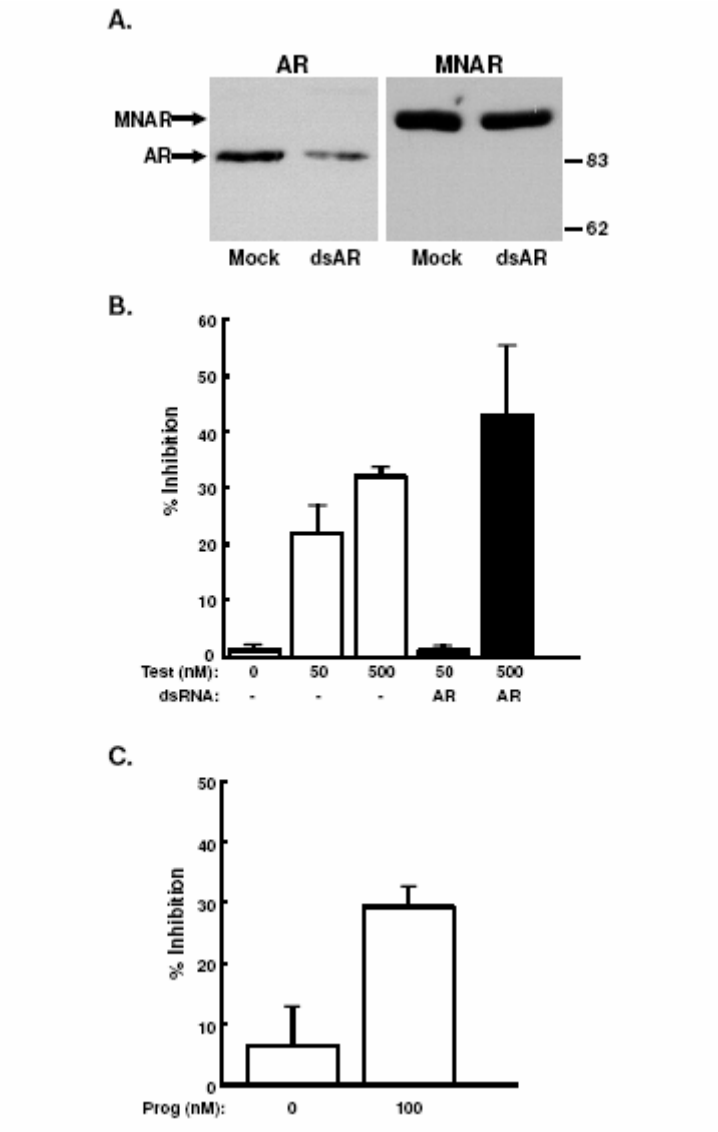

Figure 3.5. Both the AR and PR may regulate steroid-triggered inhibition of GIRK activity. A) AR expression was reduced in oocytes injected with cRNA encoding GIRK1/2 as well as double-stranded cRNA corresponding to the AR. MNAR expression was unchanged in these oocytes. "Mock" represents oocytes injected only with GIRK1/2. B) The oocytes from (A) were treated with the indicated concentrations of testosterone and potassium flux was measured after 5 minutes. C) Oocytes injected with GIRK1/2 were treated with progesterone and potassium flux measured at 5 minutes. All experiments were performed at least three times with nearly identical results, and each bar represents the mean +/- S.D. (n=3).

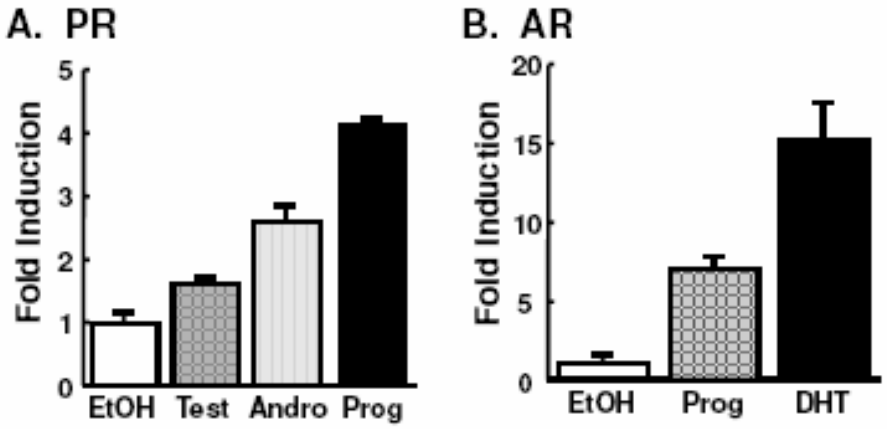

Figure 3.6. Androgens and progesterone can promote transcription via either the AR or PR. A) Androgens are weak activators of PR-mediated transcription. COS cells were transfected with an expression plasmid encoding the *Xenopus* PR, an MMTV-luciferase plasmid, and a CMV-β-galactosidase plasmid. Cells were treated with the indicated steroids at a concentration of 100 nM for 12 hours. B) Progesterone is a good activator of AR-mediated transcription. CV1 cells were transfected with an expression plasmid encoding the *Xenopus* AR, an MMTV-luciferase plasmid, and a CMV-β-galactosidase plasmid. Cells were treated with 10 nM DHT or 0.65 nM progesterone for 48 hours. In both graphs, the y-axis indicates fold-induction corrected for β-galactosidase activity (transfection efficiency), and each bar represents the mean +/- S.D. (n=3). Of note, these levels represent the maximum stimulation by the indicated steroids. Higher concentrations of steroid did not further increase transcriptional activity. (Experiment performed by Michelle Jamnongjit Ph.D.)

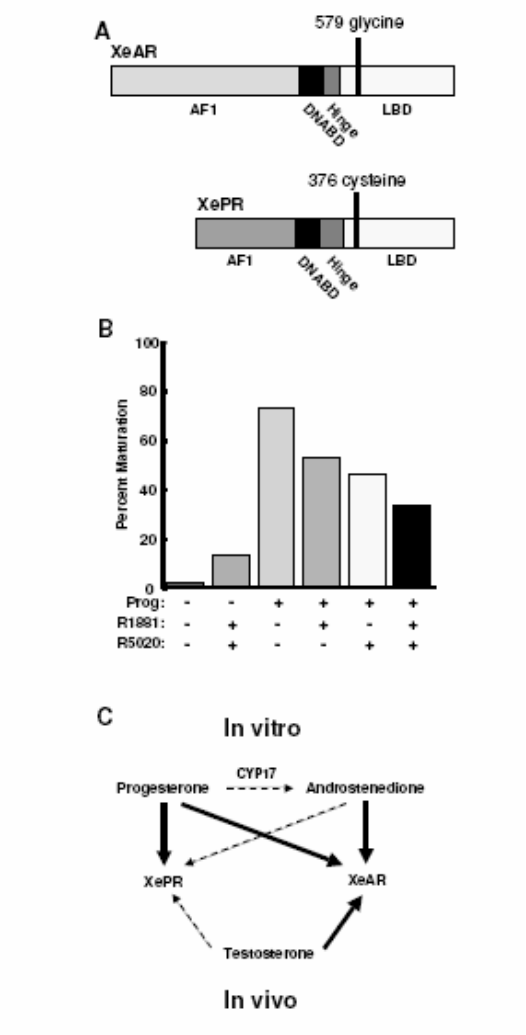

Figure 3.7. Androgens and progesterone may promote oocyte maturation via either the AR or PR. A) Schematics of the Xenopus AR and PR. The PR contains a cysteine residue at position 376, which results in decreased affinity for RU486. In contrast the AR contains a glycine residue at the equivelent position, which allows it to bind to RU486 with high affinity. B) Blockade of both the AR and PR markedly reduces progesterone-triggered oocyte maturation. Oocytes were treated with either 0.1% ethanol (-) or 100 nM progesterone plus 1 μM R1881 and/or 1 μM RU5020 (R5020) as indicated. Each bar represents the percent maturation (as determined by observing germinal vesicle breakdown) for 20 oocytes. The experiment was repeated twice with similar results. C) Model for steroid-triggered maturation of Xenopus oocytes. *In vitro*, progesterone can bind with equally high affinities to both the PR and AR, and therefore likely promotes oocyte maturation via both receptor (thick lines). In addition, progesterone is metabolized by endogenous CYP17 to androstenedione, which may activate maturation via high affinity binding to the AR or low affinity binding (thin dotted line) to the PR. *In vivo*, significant levels of progesterone are not produce in response to gonadotropin. Instead, testosterone is produced, which triggers maturation via the AR at low concentrations (due to its high affinity, thick line) and both the AR and PR at high concentrations (due to additional low affinity binding to the PR, thin dotted lines). (B. Performed by Michelle Jamnongjit Ph.D

Chapter 4

Conclusions and Future Directions

Steroid Production in the Ovary

The signaling pathways elucidated to be necessary for steroid production in the testes need to be tested for importance in the ovary. MMPs and the EGFR have already been seen to be important (8). Inhibitors directed towards specific MMPs need to be tested, such as the inhibitor for MMP2/9, to figure out which specific MMPs are important for this process. The importance of StAR has also been shown already (25-27). More research is needed to understand whether phosphorylation of StAR is necessary or if changes in total StAR are sufficient to affect steroid production. Also, positive and negative regulators or StAR can be looked at to further understand the mechanism for StAR action. cAMP has also been shown to be important, but further studies will still be helpful (16, 23, 24). It will be interesting to see if MAPK signaling plays a part in the pathway and if Src plays a role in steroidogenesis in the ovary since it did not in the testes. These studies can be performed *in vivo* with pre-pubertal pregnant mare serum gonadotropin (PMSG)-primed female mice and also *in vitro* using ovarian follicles, oocyte-granulosa cell complexes (OGCs) or oocyte-cumulus cells complexes (OCCs) as previously used to show the importance of MMPs in general and the EGFR (8).

Steroid Production in the Testes

We can look further into the physiological significance of the pathways illuminated for steroid production in the testes. The importance of cAMP, MAPK and StAR can be looked at in the more physiologically relevant systems of primary leydig

cells and *in vivo* using male mice. Again, inhibitors of these pathways will be utilized to answer these questions.

We would also like to see if PKA is directly activating the EGFR, as has been hypothesized through these studies. There are antibodies that recognize a putative phospho-PKA substrate consensus sequence. If these antibodies can recognize this sequence on the EGFR that may help provide evidence for the possibility of direct phosphorylation on the EGFR by PKA.

Ooctye Maturation in *Xenopus laevis*

Utilizing GIRKs and the two-voltage electrode clamp machine, other aspects of oocyte maturation discovered in *Xenopus laevis* can be confirmed in real-time. MNAR expression could be knocked down using RNAi or antisense oligos which should increase the severity of the decrease in potassium channel current caused by testosterone. This would help confirm in real-time what has already been shown about the importance of MNAR in holding oocytes in meiotic arrest. Another useful experiment would be to knock down GPR3 using RNAi or antisense oligos in order to see if that causes a decrease in the starting current amplitude of the GIRKs compared to mock injected oocytes. This would show that GPR3 is the G-protein or one of the G-proteins keeping oocytes in meiotic arrest. This system has proven to be a useful way to strengthen the findings about different signaling molecules involved in oocyte maturation in *Xenopus laevis* and should be useful for continuing to confirm new findings.

Implications for PCOS

These studies illuminate several possible targets for alleviating anovulation due to PCOS. The use of an EGFR inhibitor such as AG1478 or Erlotinib could help decrease improper steroid production, which leads to the excess androgen production. An EGFR kinase inhibitor could be administered for a short amount of time at a low dose until the woman is able to ovulate. Further time courses and dose responses in the ovary will be needed prior to understanding the best timing for administering an EGFR inhibitor and whether or not this will be feasible in patients suffering from PCOS. Also, further studies in the ovary are needed to figure out if inhibition of specific MMPs could be useful in allowing PCOS patients to begin ovulating.

The *Xenopus* studies showing the importance of nongenomic androgen actions via the classical AR may lead to a novel target for PCOS as well. If the nongenomic affects of the AR could be blocked to help with fertility, without affecting other actions, this may allow for less side-effects for PCOS patients. There are several synthetic selective androgen receptor modulators (SARMs), that may affect non-genomic actions of the AR without affecting transcriptionally regulated events. Several groups have synthesized SARMs that once characterized could be a possible target for PCOS patients (108, 109). *Xenopus laevis* oocytes and can be used to test out some of the SARMs for the ability to cause maturation, which we know is a nongenomic event. Also, binding studies and transcription assay using COS cells, as shown previously, can be done to figure out the affinity of different SARMS for the AR as well as the ability of each SARM to affect transcription related events. After these SARMs have been thoroughly characterized, the best ones can be looked at further for their usefulness in PCOS.

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