**DISSERTATION**

# IDENTIFICATION AND CHARACTERIZATION OF TWO OVINE MEMBRANE RECEPTORS FOR PROGESTERONE

**Submitted by**

**Ryan L. Ashley**

**Biomedical Sciences**

**In partial fulfillment of the requirements**

**for the degree of Doctor of Philosophy**

**Colorado State University**

**Ft. Collins, Colorado**

**Spring 2007**

COLORADO STATE UNIVESITY

January 14, 2007

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY RYAN L. ASHLEY ENTITLED IDENTIFICATION AND CHARACTERIZATION OF TWO OVINE MEMBRANE RECEPTORS FOR PROGESTERONE BE ACCEPTED AS FULFULLING IN PART THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Patrick D. Burns

Colin M. Clay

Gordon D. Niswender

Advisor

Terry M. Nett

Department Head

Barbara M. Sanborn

## ABSTRACT OF DISSERTATION

## IDENTIFICATION AND CHARACTERIZATION OF TWO OVINE MEMBRANE RECEPTORS FOR PROGESTERONE

Classically, progesterone (P4) has been thought to act only through the well-known genomic pathway involving hormone binding to nuclear receptors (PR) and subsequent modulation of gene expression. Alternatively, there is increasing evidence for rapid, nongenomic effects of P4 in a variety of tissues in mammals; however the receptor responsible for these actions is yet to be characterized. The likelihood of a membrane P4 receptor (mPR) causing these events is quite plausible. Sheep are the experimental model often used in our laboratory, because regulation of reproductive function is very similar in ewes and women. Nongenomic actions of P4 have been described in sheep, but the receptor(s) responsible has not been identified. The objective of this dissertation was to isolate and characterize a putative ovine membrane P4 receptor distinct from the nuclear PR. A membrane protein found to bind P4 was first isolated from porcine liver and is now known as P4 receptor membrane component 1 (PGRMC1). Since PGRMC1 is expressed in a variety of species and tissues, I hypothesized that PGRMC1 was the protein responsible for the nongenomic effects of P4 in sheep. As such, the purpose of my initial studies was to determine if PGRMC1 was expressed in the sheep and further verify if PGRMC1 was present in the plasma membrane of the ovine corpus luteum (CL). A protein lacking homology to the nPRs was isolated from the sheep and consists of 194

amino acids and contains a single transmembrane domain at the N-terminus. Despite the transmembrane domain, the ovine PGRMC1 displays predominant localization in the endoplasmic reticulum. My working hypothesis at the time was that the nongenomic effects of P4 reported in sheep coupled with the specific binding of P4 in membrane fractions from ovine CL was occurring through a unique PR at the plasma membrane. During my initial studies, another putative mPR was cloned from the seatrout that displayed seven transmembrane domains, indicative of a G-protein-coupled receptor (GPCR) and upon ligand activation, also caused rapid induction of second messenger pathways. As such, I wanted to determine if this mPR was also present in the sheep. An ovine mPR distinct from the nuclear PR was isolated and characterized. The ovine mPR is a 350 amino acid protein that, based on predicted structural analysis, possesses seven transmembrane domains and is expressed in the hypothalamus, pituitary, uterus, ovary and CL. The first characterization of mPR expression throughout the estrous cycle of the sheep, in the hypothalamus, pituitary, uterus, ovary, and CL is also reported. In CHO cells that overexpress a mPR-GFP fusion protein the ovine mPR was uniquely localized to the endoplasmic reticulum and not the plasma membrane. The ovine mPR specifically binds only progestins. Progesterone, 20$\alpha$-hydroxyprogesterone and 17$\alpha$-hydroxyprogesterone significantly ($P < 0.001$) displaced binding of $^{3}$H-P4 to membrane fractions from CHO cells expressing ovine mPR. Additionally, the ovine mPR directly induces $Ca^{2+}$ release from the endoplasmic reticulum upon ligand activation. Further, the ovine mPR appears to activate the MAPK pathway, specifically by phosphorylation of JNK1/2 upon ligand activation. This novel method of signaling at the endoplasmic

reticulum adds to the intricacy of signaling in cells and provides a mechanistically unique method for initiating actions of P4 that may alter classical dogma regarding the mechanisms by which steroid hormones act.

Ryan L. Ashley

Biomedical Sciences

Colorado State University

Fort Collins, CO 80523

Spring 2007

## ACKNOWLEDGEMENTS

I would like to begin by thanking my committee members: Pat Burns, Colin Clay, and Gordon Niswender all of whom have been extremely helpful with any questions or concerns that I had and were always willing to give up their time to assist me with anything. Next I would like to express my sincere appreciation to my advisor and mentor Terry Nett for his patience and guidance and for his tolerance for a cowboy from New Mexico learning molecular biology in his laboratory. I have learned a tremendous amount from Terry especially in the field of science and academia. I have also been very fortunate to have interacted with a great group of people at ARBL both past and present and would like to extend my thanks to all. So many people have helped me throughout my Ph.D. training and it would be impossible to thank everybody who deserves it. However, there are a few people I would like to single out. Specifically in the Nett/Niswender laboratory I would like to thank Ken and Jean Escudero for all of their help in numerous molecular techniques and more importantly for their friendship. Also I would like to thank all the graduate students and post-docs in the Nett/Niswender lab past and present (Louisa Rispoli, Alejandro Arreguin, Becky Bott, and Tracy Davis) for their help, be it bleeding sheep in the freezing cold or just simply discussing science in the lab. I have also been very privileged to have intelligent people from other laboratories at ARBL who have provided a plethora of help and knowledge. Thanks to Jeremy Cantlon, Casey Wright, Buffy Ellsworth, and Todd Farmerie. I would like to specifically thank Todd Farmerie for all of his help with the numerous cloning schemes and his overall help

with generating my fusion proteins. In particular I would also like to thank a few people who always made me laugh and made working at ARBL more enjoyable: Brian Cherrington, Amy Navratil, and Paul Gordy. Not only did these individuals provide scientific help when needed but also are just great friends. Additionally, I would like to thank my family. My parents Jeff and Ruth have always been a great support and were the first to instill upon me the importance of a good work ethic which they have and continue to demonstrate by example. Most of all I would like to thank my beautiful wife Amanda, who has been by my side all through my PhD training and has provided me with an infinite amount of support. Amanda is also a graduate student and knows first hand the ups and downs of science. Amanda has always been willing to help me with proofreading and statistical analysis, just to name a few and also has provided a tremendous amount of help with computer-related work when I have come close to throwing the computer in the Poudre! I am extremely indebted to you Amanda. Above all, I owe my entire existence to God. He has blessed me with awesome family, friends, and advisors and it is because of God that I have what I have today.

TABLE OF CONTENTS

# CHAPTER I

## LITERATURE REVIEW

### FUNCTIONS AND ACTIONS OF PROGESTERONE AND THE POSSIBLE RECEPTORS INVOLVED

Introduction: Progesterone (P4) is a steroid hormone that is synthesized by the ovary with the amount of P4 secreted depending on the level of gonadotropin stimulation and the physiological status of the ovary. Granulosa cells, theca/stromal cells and luteal cells all secrete P4 albeit at different levels (Gore-Langton and Armstrong 1988; Niswender and Nett 1988; Duleba, Spaczynski et al. 1999). Progesterone has been shown to mediate many biological effects in a variety of tissues, and as such considerable research has focused on the mechanisms through which P4 mediates its actions. Many physiological effects of P4 are mediated through gene regulation by the nuclear P4 receptors (nPR) that function as ligand-dependent transcription factors. However, not all effects of P4 are mediated by direct control of gene expression. Progesterone also has rapid stimulatory effects on the activities of a variety of signal transduction molecules and pathways. In this chapter, an overview of the functions and actions, both genomic and nongenomic, attributed to P4 will be presented as well as the possible receptors and binding proteins involved.

The multiple functions of progesterone. Progesterone plays a pivotal role in reproduction. Primary target organs of P4 include the hypothalamus, pituitary, uterus, ovary and mammary glands; moreover, effects of P4 in non-reproductive tissues are becoming more evident. The most notable role of P4 is maintaining pregnancy by stimulating secretion of uterine milk and reducing contractions of the myometrium (Senger 1999). Additionally, P4 increases synthesis of collagenase in the outer connective tissue layer of the mammalian follicle, thereby permitting ovulation to occur; is involved in structural remodeling and cellular differentiation during formation of the corpus luteum (CL); and aids in post natal mammary gland development, where it is required for mammary ductal side branching and alveologenesis (Soyal, Ismail et al. 2002). In rodents, activation of estrous behavior and the lordosis reflex is largely due to P4 sensitive neurons in the ventromedial nuclei of the hypothalamus (Pfaff, Schwartz-Giblin et al. 1994; Pfaff 1997). Upon injection of P4 directly into the brain of estrogen-primed rats, lordosis behavior is observed within 5-15 minutes (Lisk 1960; Meyerson 1972; Kubli-Garfias and Whalen 1977; McCarthy, Felzenberg et al. 1995). On the afternoon of proestrus in rats, a surge of LH, which triggers ovulation, is released in response to positive feedback actions of increasing concentrations of estradiol. This surge is further amplified by P4 (Everett 1948). However, after the LH surge, P4 exerts an inhibitory influence on GnRH pulse frequency (Kalra 1993). From these studies and others, diverse effects of P4 on the brain are evident, yet the exact mechanisms of action of P4 and specific receptor(s) responsible have not been identified.

Progesterone is also currently being studied as a neuroprotective and neuroregenerative agent in stroke and traumatic brain injuries. Progesterone appears to be quite helpful in the post-injury treatment of both male and female patients with acute traumatic brain damage (Stein 2001). Treatment with P4 also enhances recovery after stroke and spinal cord injuries. Thomas and coworkers created contusion injuries of the spinal cord in male rats and then treated them with one injection/day of P4 for 5 days (Thomas, Nockels et al. 1999). These researchers noted that P4 reduced the lesion size and prevented additional secondary neuronal loss. By the end of 6 weeks of behavioral testing, rats given P4 showed significantly better hindlimb and forelimb coordination and walked better than their untreated counterparts.

In addition to steroidogenic tissues, P4 is also produced by Schwann cells where it increases formation of new myelin sheaths after lesion of the rodent sciatic nerve (Baulieu and Schumacher 2000). Furthermore, blocking local synthesis or action of P4 revealed impaired remyelination of the regenerating axons, whereas administration of P4 to the lesion site promoted formation of new myelin sheaths. Therefore, it appears P4 may prove effective as a novel treatment for degenerative disorders of the nervous system.

Much controversy exists regarding P4 and various reproductive cancers. Because of the influence of ovarian function on breast cancer, there has been extensive examination of a possible correlation between hormone therapy (P4 and estradiol) and breast cancer risks. To date, there is no definitive evidence that P4 acts in the pathogenesis of breast cancer. In contrast, P4 reduces the risk of developing ovarian

carcinoma (Key 1995) and can activate p53 expression, inhibit growth, and cause apoptosis in ovarian and breast cancer cells (Bu, Yin et al. 1997; Formby and Wiley 1999).

Genomic actions of progesterone. From the above, it is clear that P4 exerts numerous actions on various tissues, yet the precise mechanism by which P4 causes many of these actions remains an enigma. Progesterone alters transcription of a variety of genes in target tissues through the well-known genomic pathway involving hormone binding to nuclear P4 receptors (nPR) and subsequent modulation of gene expression (Beato 1989). These specific intracellular receptors are members of the nuclear/intracellular transcription factors (Tsai and O'Malley 1994; Leonhardt SA, Boonyaratanakornkit V et al. 2003). Following binding of P4 to its receptor, a conformational change occurs in the nPR and the receptor ligand complex is translocated to the nucleus where it interacts with DNA binding elements and alters the transcription of P4 responsive genes (Ismail, Amato et al. 2003).

The genomic organizations of these nPRs are fairly well characterized. Nuclear PRs contain three functional domains, the N-terminus, the DNA-binding domain (DBD) and the C-terminal, ligand-binding domain (LBD). Activation domains present within these functional complexes are required for the DNA bound receptor to transmit a transcription activation response and these sites also serve as specific binding sites for co-activators. The N-terminal domain is required for the full transcriptional activity of the receptor and for other cell and target gene specific responses (Leonhardt, Boonyaratanakornkit et al. 2003). This domain contains the transcription activation

domains AF-1 and AF-3 that recruit co-activator proteins to the receptor and thereby regulate promoter specificity and also transcriptional activity (Conneely, Mulac-Jericevic et al. 2003).

The DBD is a highly conserved, centrally located domain consisting of approximately 66-68 amino acids (Conneely, Mulac-Jericevic et al. 2003). Two type II zinc finger structures in this domain facilitate the binding of the receptor to specific cis-acting DNA structures (Freedman 1992). The DBD also contains a nuclear localization sequence (NLS), dimerization domain (DI) and a highly conserved AF-2 domain.

The LBD is involved in binding P4 and heat shock proteins. It contains another determinant of dimerization, which functions in the absence of DNA binding and a second NLS. Progesterone binding induces a conformational change in the receptor that creates a hydrophobic binding pocket of the p160 family of steroid receptor co-factors, which then activates AF-2 (Leonhardt, Boonyaratanakornkit et al. 2003). This conformational change also leads to phosphorylation of the receptor and dissociation from heat shock proteins and dimerization. The ligand receptor complex then enters the nucleus and binds and activates specific response elements.

Three isoforms of nPRs are currently known, namely PR-A (94 kDa), PR-B (116 kDa) and PR-C (60 kDa). PR-A and PR-B are translated from the same gene; however, the two isoforms are produced through alternate use of two promoters that give rise to two different PR mRNAs (Hanekamp, Gielen et al. 2003). PR-B contains an additional 164 amino acids at the N-terminal domain called the B-upstream sequence (Kastner, Krust et al. 1990). In tissue culture, PR-A and PR-B exhibit different properties that are

specific to particular cell types. In most cases, PR-B is a stronger activator of transcription of several target genes regulated by both receptors (Richer, Jacobsen et al. 2002). When PR-A and PR-B are coexpressed in cells where the endogenous PR-A is inactive as a transcriptional activator, the expressed PR-A functions as an extremely potent, ligand-dependent repressor of PR-B transcriptional activity (Vegeto, Shahbaz et al. 1993). Additionally, PR isoforms display a different response to progestin antagonists. While antagonist bound PR-A is inactive, antagonist bound PR-B can be converted to a powerfully active transcription factor (Conneely and Lydon 2000).

PR-C is smaller than the other two isoforms, truncated at the N-terminal domain and known to have unique transcriptional potentiating properties, although its progestin and antiprogestin binding is similar to PR-A and PR-B. The dissociation constant (Kda) however, for PR-C is five times higher than PR-A and PR-B, and it has been reported that PR-C enhances the transcriptional activity of PR-B (Wei, Norris et al. 1997).

The pathway by which these nPRs function is often called "genomic" because it involves direct action on the genome. One noticeable attribute of genomic mechanisms is the length of time to elicit a physiological response. Given the length of time between steroid entry and the accumulation of significant amounts of newly formed protein, this process often requires hours for an overall physiological response. In contrast, numerous reports exist in which P4 causes physiological responses within seconds to minutes and these actions are termed nongenomic.

Nongenomic actions of progesterone. The first nongenomic effect of P4 was described by Dr. Hans Selye (Selye 1942) in which anesthetic effects occurred almost

immediately after application of P4 to rats. Since these initial studies, much research has been conducted on nongenomic effects of P4 as well as other steroids. Nongenomic P4 actions have been demonstrated in various tissues including brain, uterus, oocytes, sperm, Leydig cells, and vasculature with varying physiological responses.

One of the most thoroughly studied systems of rapid signaling by P4 is induction of *Xenopus* oocyte maturation. *Xenopus* oocytes naturally arrest at the $G_2$ phase of meiosis I. Treatment of *Xenopus* oocytes with P4 causes a rapid decrease in cAMP and a decline in protein kinase A (PKA) activity that leads to activation of a MAPK pathway and maturation-promoting factor, causing germinal vesicle breakdown and resumption of meiosis II (Maller 2001). Whether the drop in cAMP/PKA is sufficient for induction of intracellular signaling pathways and oocyte maturation is not known. Use of cAMP analogs or phosphodiesterase inhibitors to maintain elevated levels of cAMP blocks oocyte maturation, whereas inhibitors of PKA can induce oocyte maturation in the absence of P4 (Lutz, Kim et al. 2000; Duckworth, Weaver et al. 2002). Lutz and coworkers suggest that P4 induction of oocyte maturation is caused by a release from cAMP/PKA inhibition mechanism by P4 relieving a constitutive inhibitory signal by $G\beta\gamma$ subunits (Lutz, Kim et al. 2000; Duckworth, Weaver et al. 2002).

Progesterone also causes rapid effects on second messengers and the acrosome reaction in human sperm. The acrosome reaction is a modified exocytotic event that facilitates penetration of the spermatozoa into the ovum and the sperm-egg plasma membrane fusion required for fertilization. Progesterone is secreted in high concentrations into the cumulus oophorous and follicular fluid surrounding the egg and is

an initiator of the acrosome reaction by acting in a nongenomic manner as determined by the fact that nuclei of spermatozoa are transcriptionally inactive (Nemere, Pietras et al. 2003). Progesterone stimulates a rapid influx of extracellular calcium ($Ca^{2+}$) and an efflux of chloride ions, both of which are essential for induction of the acrosome reaction (Meizel, Turner et al. 1997). Further, a $Ca^{2+}$-dependent increase in cAMP and phosphorylation of several spermatozoal proteins are also involved in mediating the P4-initiated acrosome reaction, and studies using inhibitors have implicated a role for both PKC and cAMP-dependent PKA. PKA and A kinase anchoring proteins (AKAP) are expressed in the acrosomal region of sperm and specific inhibitors of PKA and peptides designed to disrupt PKA-AKAP interactions inhibit the P4-initiated acrosome reaction (Harrison, Carr et al. 2000). Progesterone also activates other signal transduction pathways in human sperm, such as phospholipase C (PLC) (Thomas and Meizel 1989) and a tyrosine kinase (Tesarik, Moos et al. 1993).

Nongenomic effects of P4 have also been demonstrated in rat Leydig cells (Rossato, Nogara et al. 1999). Specifically, P4 was able to induce depolarization of the plasma membrane. This depolarization was due to an influx of $Na^{+}$ from external medium as depolarization was absent when extracellular $Na^{+}$ was iso-osmotically substituted with choline or sucrose. In contrast to sperm, no effect on intracellular $Ca^{2+}$ at any dose of P4 tested was observed in Leydig cells. Additionally, in Leydig cells, P4 conjugated to bovine serum albumin (P4-BSA) was able to stimulate steroidogenesis in a dose-dependent manner and these researchers also identified a membrane binding site for P4 in rat Leydig cells. As the nuclear P4 receptor does not appear to exist in these cells

(Due, Dieckmann et al. 1989), the possibility exists for a membrane PR which would account for the rapid P4 actions noted in rat Leydig cells.

Progesterone in both the ventromedial hypothalamus (VMH) and the ventral tegmental area (VTA) is necessary to facilitate sexual receptivity in estrogen-primed hamsters. The mechanism of action for P4 however, appear to be different in the VMH versus VTA as the VMH has many nPRs but the VTA has few, yet both areas respond to P4. Application of P4-BSA to VTA rapidly increases sexual receptivity if P4 has been applied earlier to the VMH (DeBold and Frye 1994). P4-BSA is ineffective when applied to the VMH. These data are consistent with P4 facilitation of sexual receptivity being genomically mediated in the VMH, while the actions of P4 mediated at the VTA are nongenomic. In support of this notion, within 60 seconds of intravenous or intra-VTA administration of P4 to hamsters and rats there are increases in neuronal firing in the VTA (Rose 1990; Frye, Bayon et al. 2000). Furthermore, rapid effects of P4 and P4-BSA have been described in superfused slices of the rat mediobasal hypothalamus (Drouva, Laplante et al. 1985; Ke and Ramirez 1987).

The effects of P4 on uterine smooth muscle are numerous. In addition to genomic effects, P4 may promote uterine relaxation by nongenomic mechanisms. Nongenomic effects of acute P4 exposure include inhibition of transmembrane $Ca^{2+}$ entry, release of $Ca^{2+}$ from intracellular stores (Kosterin, Burdyga et al. 1994) and membrane hyperpolarization with subsequent activation of $K^{+}$ channels (Mironneau, Savineau et al. 1981). Another nongenomic effect at the uterus has been reported by Grazzini et al., (Grazzini, Guillon et al. 1998) who demonstrated that P4 inhibited oxytocin binding to

rat oxytocin receptors located on the plasma membrane. Similar results have also been demonstrated in cattle (Bogacki, Silvia et al. 2002) and sheep (Dunlap and Stormshak 2004). These studies strongly support the possibility of a membrane P4 receptor (mPR) in the uterus which may interact with the oxytocin receptor.

Nongenomic effects of P4 on the vasculature have been reported in a variety of species. In non-human primates, intracoronary administration of P4 causes direct vasorelaxation of constricted arteries within minutes of treatment (Minshall, Pavcnik et al. 2002). Similar vasorelaxation was induced by P4 in rabbit coronary arteries (Jiang, Sarrel et al. 1992) and in rat aorta. The vasorelaxation appeared to be mediated by inhibition of $Ca^{2+}$ entry or blockage of voltage-dependent and/or receptor-operated $Ca^{2+}$ channels (Glusa, Graser et al. 1997). Additionally, P4 can rapidly reduce arterial pressure in sheep (Roesch and Keller-Wood 1997). These researchers demonstrated that physiological levels of P4 decreased arterial pressure within 17 min with no evidence of changes in plasma electrolytes or volume over the 120 min study. The specific mechanism by which P4 decreases systemic pressure is not known currently.

Since, the ovary synthesizes P4 in high amounts, it has been difficult to determine whether P4 influences ovarian function. However, as early as 1981 Rothchild proposed that P4 has an intraovarian site of action based on its ability to regulate its own secretion in luteal cells (Rothchild 1981). Recently, more studies have investigated the rapid or nongenomic effects of P4 in the ovary, specifically the role of $Ca^{2+}$ in the regulation of steroiogenesis and secretion of P4 by granulosa cells. In pig granulosa cells, P4 stimulates the formation of inositol-1,4,5-triphosphate and increases the intracellular $Ca^{2+}$

concentration via mobilization of $Ca^{2+}$ from the endoplasmic reticulum (Machelon, Nome et al. 1996). It was also demonstrated that the increase in $Ca^{2+}$ occurred due to activation of phospholipase C linked to a pertussis-insensitive G-protein, suggestive of a G-protein-coupled-receptor (GPCR), specifically of the $G_q$ family (Machelon, Nome et al. 1996). Similar results have been reported in human granulosa-lutein cells in which P4 caused an increase in intracellular $Ca^{2+}$ within seconds of treatment (Younglai, Wu et al. 2005). Progesterone also causes rapid changes in intracellular $Ca^{2+}$ in human intestinal smooth muscle cells (Bielefeldt, Waite et al. 1996), T lymphocytes (Ehring, Kerschbaum et al. 1998), oocytes (Moreau, Vilain et al. 1980; Wasserman, Pinto et al. 1980) and spermatozoa (Thomas and Meizel 1988; Osman, Andria et al. 1989; Baldi, Casano et al. 1991; Foresta, Rossato et al. 1992; Krausz, Bonaccorsi et al. 1995).

Potential mediators of progesterone's nongenomic effects. From the above it is evident that P4 causes numerous nongenomic responses in a variety of tissues. It seems very unlikely that P4 can cause such rapid cellular changes via the classic genomic mechanism that normally requires several hours for an effect to be observed. Recently, there have been major advances in identifying putative P4 receptors that transduce P4's actions independent of the genomic action of the nPRs. These rapid responses could be activated through P4 binding to either nPRs localized at or near the plasma membrane or to various membrane P4 receptors or binding proteins that have been reported. The remainder of this chapter will provide a description of the proteins thought to be involved in mediating nongenomic actions of P4.

In addition to the genomic effects initiated by the nPRs, rapid P4 activation of the Src/Ras/MAPK pathway in the absence of transcription has been shown to be dependent on the classical nPRs (Migliaccio, Piccolo et al. 1998). These researchers suggested that nPR activation of Src/Ras/MAPK pathway was indirect through nPR association with the estrogen receptor (ER) and that it was ER that directly activated Src through interaction with the Src homology 2 domain (SH2 domain). This dependency on ER for progestin activation of Src however, was observed only in transiently transfected Cos-7 cells. Further, whether nPR and ER can physically interact is controversial and how ER interaction with nPR can activate Src has not been shown (Migliaccio, Piccolo et al. 1998).

Recently, another mechanism for how nPR interacts with and activates Src and the downstream MAPK pathway was reported (Boonyaratanakornkit, Scott et al. 2001). The N-terminal region common to both PR-A and PR-B contains a short contiguous polyproline sequence (amino acids 421-428, Pro-Pro-Pro-Pro-Leu-Pro-Pro-Arg) that conforms to a consensus type II, Pro-Xaa-Xaa-Pro-Xaa-Arg (PXXPXR), motif for binding the SH3 domain of cytoplasmic signaling molecules. These polyproline sequences form a left-handed helix that interacts with the binding pocket of SH3 domains. Through this PXXPXR motif, the nPR interacts directly with the SH3 domain of various signaling molecules, including Src (Boonyaratanakornkit, Scott et al. 2001). Further, the nPR interaction with the SH3 domain of Src through the PXXPXR motif also occurs in cells in a P4-dependent manner.

Interaction of nPR with SH3 domains and activation of Src appear to be of physiological importance in mammalian cells. Progesterone-induced activation of the entire Src/Ras/MAPK signaling pathway in mammalian cells is dependent on the integrity of PXXPXR motif in nPR and the ability of nPR to interact with the SH3 domain of Src. Also, the nPR-SH3-domain interaction contributes to P4-induced inhibition of cell proliferation and cell cycle progression of normal mammary epithelial cells in culture. Interestingly, point mutations in the PXXPXR motif that abolish P4-induced Src activation have no effect on the transcriptional activity of the nPR. Thus, despite the point mutation which abolishes Src activation by the nPR, it still operates as a transcription factor. On the other hand, point mutations in the DNA binding domain or AF-2 that compromise nPR function as a transcription factor do not interfere with the ability of nPR to mediate P4-induced activation of Src (Boonyaratanakornkit, Scott et al. 2001).

These data are suggestive of the nPR mediating both transcriptional and nontranscriptional actions which are separable. As such, it appears the nPRs are dual-functioning proteins capable of directly interacting with DNA in the nucleus and functioning in its well-established role as a transcription factor, and interacting with SH3 domains of Src and perhaps other signaling molecules to modulate intracellular signaling pathways.

In addition to the nPRs (PR-A and PR-B) several groups have reported the existence of N-terminally truncated forms of nPR or gene products closely related to nPRs as detected by immunoblot with monoclonal antibodies to the C-terminal tail of

human nPRs. These particular antibodies recognize an epitope in the LBD of nPRs that is conserved among species. Given that the various N-truncated nPRs often lack a functional DBD, yet still posses a LBD for P4, it is often thought they may function as nongenomic receptors. One such protein has already been mentioned in this review and is referred to as PR-C. This isoform of nPR (PR-C) was identified in breast cancer cells and arises through an alternate use of an internal ATG codon located within the DBD of PR-A and PR-B to generate a PR protein lacking the N-terminal domain, specifically the first zinc finger of the DBD but still containing a complete LBD. The PR-C protein as mentioned lacks a functional DBD, is inactive as a transcription factor but retains normal hormone binding activity and can modulate the transcriptional activity of PR-A and PR-B (Wei, Hawkins et al. 1996). Whether PR-C functions in a nongenomic manner is currently not known.

A unique PR mRNA has been identified in a testis cDNA library. The message contains an additional novel 5' exon (termed exon S), lacks the N-terminal domain, yet retains exons 4-8 of the nPR (Hirata, Shoda et al. 2000). Expression of PR-S was found in spermatozoa and uterus with a higher expression level in the former. However, the S form of PR has been detected only as an RNA transcript. The PR-S isoform protein has not been detected nor have studies been completed to explore a functional role for PR-S. Another truncated PR reported recently was cloned from both human adipose and aortic cDNA libraries (Saner, Welter et al. 2003). This PR, termed PR-M, lacks the amino terminal A/B regions and the DBD. An intriguing aspect of PR-M is the existence of a signal peptide on the amino-terminus which the authors suggest may be utilized for

membrane localization. Amino acid sequence after the signal peptide is homologous with nPRs from exon 4 to the end of the protein. The function of PR-M remains to be determined.

The first protein lacking homology to the nPRs to be isolated from a membrane fraction and bind P4 was identified in porcine liver membranes (Meyer, Schmid et al. 1996). These researchers discovered that fractions revealing specific binding of P4 contained two major polypeptides of apparent molecular masses of 28 and 56 kDa, respectively, representing the first membrane steroid binding protein for which both biochemical and sequence information is available (Meyer, Schmid et al. 1996). The larger molecular mass protein is thought to represent a dimer. This protein, now referred to as P4 receptor membrane component 1 (PGRMC1) (Losel, Dorn-Beineke et al. 2004) is composed of 194 amino acids and possesses a single membrane-spanning domain (Falkenstein, Meyer et al. 1996; Meyer, Schmid et al. 1996). Binding studies indicate that PGRMC1 has a high affinity ($k_d$ = 11 nM) and a low affinity ($k_d$ = 286 nM) binding site for P4 (Falkenstein, Meyer et al. 1996; Meyer, Schmid et al. 1996). Further, PGRMC1is expressed in a variety of tissues and species. Tissues found to express PGRMC1in the pig include the liver, kidney, lung, cerebellum, spleen, heart (Falkenstein, Meyer et al. 1996) and sperm (Losel, Dorn-Beineke et al. 2004). Also, mRNA encoding PGRMC1 is detected in preovulatory mouse follicles (McRae, Johnston et al. 2005), porcine granulosa cells (Jiang, Whitworth et al. 2004), human granulosa/luteal cells maintained in cluture (Sasson, Rimon et al. 2004) and bovine lens epithelial cells (Zhu, Sexton et al. 2001).

Although PGRMC1 has been implicated as a mediator of P4's action in sperm (Losel, Dorn-Beineke et al. 2004), virtually nothing is known about the signal transduction pathways that are activated when it binds P4. Interestingly, PGRMC1 has a very short cytoplasmic domain, which has several potential SH2 and SH3 domains. Whether these domains are involved in the transduction of the ligand-activated signal associated with PGRMC1 remains to be determined.

Recent research by Peluso and colleagues has added another twist to PGRMC1's possible functions. These researchers demonstrated that PGRMC1 interacts with Plasminogen Activator Inhibitor RNA binding protein-1 (PAIRBP1), a membrane-associated protein involved in the anti-apoptotic action of P4 (Peluso, Pappalardo et al. 2006). Localization by confocal microscopy revealed both proteins colocalize to the plasma membrane as well as the cytoplasm. Additioanlly, PGRMC1 and PAIRBP1 were identified in rat spontaneously immortalized granulosa cells as well as granulosa and luteal cells. Overexpression of PGRMC1 increased $^{3}$H-P4 binding, whereas treatment with a PGRMC1 antibody blocked P4's anti-apoptotic action. Peluso and coworkers suggest that at the plasma membrane, PGRMC1 and PAIRBP1 interact to form a complex that is required for P4 to transduce its anti-apoptotic action (Peluso, Pappalardo et al. 2006).

Recently, a novel G-protein-coupled receptor (GPCR) was cloned from sea trout ovaries and characterized by a receptor-capture assay. A partially purified putative membrane receptor was used to generate monoclonal antibodies which in turn were used to screen a sea trout ovaraian expression cDNA library (Zhu, Rice et al. 2003). Strong

evidence exists to support this protein as a bona fide membrane P4 receptor (mPR) that is structurally unrelated to the classic nPR. Recombinant mPR expressed in *Escherichia coli* specifically binds progestins in vitro with a relatively high affinity ($k_d$ = 30 nM), consistent with physiological concentrations of P4 in sea trout ovaries. Deduced amino acid sequences from the sea trout mPR cDNA predicted seven transmembrane domains, a characteristic of GPCRs, but not of nPRs.

To date three isoforms of this mPR have been identified (i.e., mPR$\alpha$, mPR$\beta$, and mPR$\gamma$) (Zhu, Bond et al. 2003). Expression of the $\alpha$ isoform is predominately in reproductive tissues, whereas the $\beta$ isoform is confined mainly to the brain. The $\gamma$ isoform is primarily expressed in kidney, lung and colon. Homologous transcripts for these isoforms were also confirmed in mammals, specifically human, mouse, pig (Zhu, Bond et al. 2003) and rat (Cai and Stocco 2005).

Ligand activation of the sea trout mPR$\alpha$ causes activation of the MAPK pathway (Erk 1/2) within 5 min in transfected cells and decreases in levels of cAMP (Zhu, Rice et al. 2003). This mPR-mediated inhibition of adenylate cyclase by P4 can be prevented by treatment with pertussis toxin. This is consistent with coupling of the receptor to a G-protein, specifically to $G_{i/o}$ proteins (Zhu, Rice et al. 2003). These researchers suggest mPR$\alpha$ mediates progestin induced meiotic maturation of the spotted seatrout oocyte.

As these novel mPRs were first isolated from seatrout, the majority of research pertaining to possible functions of these receptors has been performed in non-mammalian species. However, all three mPR isoforms are expressed within the rat ovary and

amounts of both mPR$\alpha$ and mPR$\gamma$ are increased in corpora lutea (CL) throughout pregnancy, while amounts of mPR$\beta$ remained constant (Cai and Stocco 2005). Specific functions of the mPRs in the rat ovary are currently not known. Both mPR$\alpha$ and mPR$\beta$ were recently reported in human myometrium and found to be differentially modulated during labor and by P4 and estradiol in vitro (Karteris, Zervou et al. 2006). Activation of the mPRs decreased levels of cAMP and increased phosphorylation of myosin light chains, both of which facilitate myometrial contraction. Despite these recent studies, very little is known about the function(s) of the mammalian mPRs.

From the reported studies it is obvious that P4 exerts numerous nongenomic effects through a variety of known receptors and binding proteins. Likewise, nongenomic effects of P4 have been reported in sheep, which are the experimental model most often used in our laboratory. Progesterone can exert a powerful inhibition on pulsatile GnRH release from the hypothalamus and consequently, LH release from the pituitary (Goodman, Bittman et al. 1981; O'Byrne, Thalabard et al. 1991). Studies investigating the effects of P4 on GnRH release have revealed rapid inhibition of GnRH secretion in ewes upon P4 treatment (Skinner, Evans et al. 1998). Due to the rapid nature of these P4 invoked responses that occur within minutes, they are considered nongenomic. The nPRs have been identified in the preoptic area, ventrolateral region of the ventromedial nucleus and the arcuate nucleus of the sheep, but whether the nPR is solely responsible for these nongenomic actions remains controversial as localization of nPRs did not colocalize with GnRH neurons (Skinner, Caraty et al. 2001). Further, specific binding sites for P4 have been identified in membrane fractions of ovine CL

(Bramley and Menzies 1994), but the protein responsible for the P4 binding has yet to be elucidated. It is very possible that a mPR unlike the nPRs exists in the sheep which accounts for many of these nongenomic actions of P4. As such, I hypothesized that a mPR was responsible for the P4 binding in membrane fractions of CL. The focus of the work in this dissertation was to isolate and characterize an ovine mPR distinct from the nPR. Two different ovine membrane proteins were identified and cloned and details of these studies will be discussed at length in this dissertation. The first protein is homologous to PGRMC1 and the second is homologous to mPRα.

Understanding the mechanism(s) of nongenomic P4 action mediated by these ovine membrane proteins will provide a sound molecular basis for future studies in this emerging field of reproductive endocrinology. Study of these proteins may lead to exciting advances in understanding the P4 dependent processes of ovulation, luteinization, follicular atresia, and maternal recognition of pregnancy, and may lead to development of novel methods of manipulating ovarian activity and fertility in humans and other mammalian species.

# CHAPTER II.

## IDENTIFICATION AND CHARACTERIZATION OF THE OVINE PROGESTERONE RECEPTOR MEMBRANE COMPONENT-1 (PGRMC1)

Introduction:

In 1942, Hans Selye published a study on the correlation between chemical structure of steroids and their pharmacological action (Selye 1942). In this study, rats were administered a number of different steroids intraperitoneally and rapid anesthetic effects were observed. This study is often considered the first evidence of nongenomic steroid actions. Since these initial experiments, numerous studies have investigated the nongenomic effects of steroids. Specifically for P4, nongenomic actions have been described in a variety of tissues, such as oocytes (Godeau, Schorderet-Slatkine et al. 1978), sperm (Thomas and Meizel 1989; Blackmore, Beebe et al. 1990; Blackmore and Lattanzio 1991), hypothalamus (Goodman, Bittman et al. 1981; O'Byrne, Thalabard et al. 1991), and uterus (Kosterin, Burdyga et al. 1994).

Since a physiological response produced by genomic mechanisms likely requires hours (Beato 1989) extensive research investigating possible mechanisms that could account for the rapid nongenomic actions has been conducted. A membrane protein found to bind P4 was first isolated from porcine liver (Falkenstein, Meyer et al. 1996; Meyer, Schmid et al. 1996) and is now known as P4 receptor membrane component 1

(PGRMC1) (Losel, Dorn-Beineke et al. 2004). This protein is not homologous to the nPR, is composed of 194 amino acids, and possesses a single membrane-spanning domain (Falkenstein, Meyer et al. 1996; Meyer, Schmid et al. 1996). Binding studies indicate that PGRMC1 has a high affinity ($k_d$ = 11nM) and a low affinity ($k_d$ = 286 nM0 binding site. Tissues found to express PGRMC1in the pig include the liver, kidney, lung, cerebellum, spleen, heart (Falkenstein, Meyer et al. 1996) and sperm (Losel, Dorn-Beineke et al. 2004). Also, mRNA encoding PGRMC1 is detected in preovulatory mouse follicles (McRae, Johnston et al. 2005), porcine granulosa cells (Jiang, Whitworth et al. 2004), human granulosa/luteal cells maintained in culture (Sasson, Rimon et al. 2004) and bovine lens epithelial cells (Zhu, Sexton et al. 2001).

Sheep are the experimental model often used in our laboratory, because regulation of ovarian function is very similar in ewes and women. As described in Chapter 1, nongenomic actions of P4 have been described in sheep, but the receptor(s) responsible has not been identified. Of particular interest to me was the report of specific binding of P4 in membrane fractions from ovine corpora lutea (CL) (Bramley and Menzies 1994). Since PGRMC1 is expressed in a variety of species and tissues, I hypothesized that PGRMC1 was the protein responsible for the P4 binding in membrane fractions of CL. As such, the purpose of this study was to determine if PGRMC1 was expressed in the sheep and further verify if PGRMC1 was present in the plasma membrane of the ovine CL.

Materials and Methods

PCR of partial sequence for ovine PGRMC1: Primers were designed based on homology between pig and human PGRMC1 (Falkenstein, Meyer et al. 1996; Bernauer, Wehling et al. 2001). The sense primier for ovine PGRMC1 was 5' ATGGCNGCNGARGAYGTNGCN 3' and anti sense primer for ovine PGRMC1 was 5' NGGNCCATARAAYTTNCKNCCYTT 3'. Ovine genomic DNA served as the template for PCR. The PCR consisted of 35 cycles with the following conditions: 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. Amplified DNA was analyzed on a 1.5% agarose gel and visualized via UV illumination. The PCR product was excised and purified from the agarose using a QIAEX II Gel Extraction Kit (QIAGEN, Valencia, CA) and subsequently ligated into pGEMT-Easy vector (Promega, Madison, WI) per the manufacture's instructions. Identity of the cDNA sample was confirmed by sequencing (UC Davis, Davis, CA).

Screening of ovine CL d-10 cDNA library for full length ovine PGRMC1: For these experiments, an ovine CL d-10 cDNA library that was generated in the laboratory of Dr. Gordon Niswender was employed. The cDNA library contains ~ 2 x $10^9$ λ/mL. Aliquots of the cDNA library were diluted with sterile SM buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgSO_4$ and 0.01% w/v gelatin) in sterile 15 mL conical tubes. To the diluted aliquots of the cDNA library, 100 µL XL1-Blue cells resuspended in 10 mM $MgSO_4$ (final dilution = $1:10^5$) was added. Conical tubes were placed in a 37°C water bath and gently shook for 20 min. Sterile top layer agarose (4 mL) (LB media, 0.72% agarose and 10 mM $MgSO_4$) was added to each conical tube, mixed and decanted onto

pre-warmed LB-plates. Plates were allowed to cool at room temperature for ~ 10 min and then inverted and incubated at 37°C overnight. Plates were then removed from the incubator and visualized for plaques. Nitrocellulose membranes (Hybond-N; Amersham Biosciences, Pittsburgh, PA) were placed on the top agarose layer for 1-3 min and marked with India ink for orientation. Membranes were carefully removed and floated for 30 sec with DNA side up in denaturing solution (1.5 M NaCl and 0.5 M NaOH) and then immersed for 60 sec. Nitrocellulose membrane were then immersed in neutralization solution (1.5 M NaCl, 0.5 M Tris, pH = 8.0) for 5 min. Membranes were then washed in 3X SSC (Na-Citrate and NaCl), air dried on Whatman filter paper and crosslinked in a CL-1000 Ultraviolet Crosslinker to fix the DNA to the filter paper.

Nitrocellulose membranes were positioned in hybridization cyclinders with hybridization solution (50% Formamide, 5X Denhardts, 100 μg/mL salmon sperm DNA (boiled for 5 min before addition to solution), 6X SSC, 25 mM EDTA and 1% SDS) and loaded into a HB-1D Hybridiser (Techne) at 42°C for 1-4 h. The isolated PCR product containing partial sequence for the ovine PGRMC1 was radiolabeled with $^{32}P$ for use as a probe. The probe was synthesized by boiling isolated ovine PGRMC1 PCR product (50 ng) plus water (final volume 25 μL) for 5 min and subsequently placed on ice. To the DNA, 10 μL 5X labeling buffer, 6 μL of 0.5 M dGATPs, 2 μL acetylated BSA, 5 μL $^{32}P$-dCTP and 2 μL Klenow were added and the mixture was incubated for 5 h at 37°C. To remove unincorporated nucleotides, radiolabeled probe was passed through a spin column (Centri-Spin; Princeton Separations, Adelphia, NJ). The DNA probe was denatured by boiling for 5 min and then added to hybridization solution. The

radiolabeled probe was then added to nitrocellulose membranes in hybridization cylinders ($10^6$ cpm/cylinder) and allowed to incubate at 42°C overnight. Next, membranes were washed at room temperature on a plate rocker for 1 h in 2X SSC + 0.05% SDS and then for 1 h in 1X SSC + 0.1% SDS. Membranes were then allowed to dry briefly on filter paper and subsequently covered with clear plastic wrap. Next, membranes were placed in a cassette and exposed to film (Kodak Scientific Imaging Film) overnight at -80°C. The cassette was removed from the freezer and allowed to reach room temperature before the film was developed. Film was aligned with filters to identify positive plaques. Positive agar plugs were picked and placed in 1 mL SM buffer containing ~ 40 µL chloroform, shook at room temperature for 4 h and then stored at 4°C. From each primary plaque that was isolated, serial dilutions were generated ($10^{-3}$, $10^{-4}$ and $10^{-5}$) in SM buffer. To each dilution of primary plaques, 100 µL XL1 bacteria were added and incubated for 20 min at 37°C with shaking. All positive plaques were subjected to two additional rounds of screening as described for the primary screen.

Isolation of DNA from lambda ZAP phage: To a 50 mL conical tube, 200 µL XL1-Blue cells, 250 µL phage and 1 µL ExAssist helper phage were added and allowed to incubate for 15 min at 37°C. Next, LB-media (3 mL/tube) was added and incubated for 2.5 h at 37°C and then for 15 min at 70°C. Samples were then centrifuged at 4000 x g (Sorvall RC-5B) for 15 min at 4°C and supernatants decanted into sterile conical tubes. Aliquots of supernatant (100 µL) were then added to 200 µL SOLR cells and incubated for 15 min at 37°C. Next, aliquots of each sample (50 µL) were dispersed onto LB-ampicillin plates and incubated overnight at 37°C. Colonies from each plate were

isolated and grown in 2 mL LB media plus ampicillin overnight at 37°C. The QIAprep Spin Miniprep (QIAGEN, Valencia, CA) was employed per manufacture's instructions to isolate and purify the pBluescript SK plasmid from the λZAP phage. Aliquots of isolated DNA were digested with *Eco*R1 and *Xho*1 restriction enzymes to screen for plasmids containing the gene of interest. Identity of cDNA samples was confirmed by sequencing (UC Davis, Davis, CA).

Preparation of ovine PGRMC1-green fluorescent protein (PGRMC1-GFP) fusion vector: cDNA encoding GFP was fused to the C-terminal of the ovine PGRMC1 by PCR. cDNA containing the full coding sequence of the PGRMC1 in pBluescript SK, with a gene-specific primer that inserted an *Eco*R1 site (underlined) upstream of the transcription start site (CGGAATTCATGGCTGCCGAGGATGTGG) and a gene-specific primer that eliminated the stop codon and substituted a *Bam*H1 site (underlined) at its 3'-end (GCGGGATCCCATCATTCTTCCGAGTGCT) was used to create in-frame restriction sites. The following PCR conditions were used: 94°C for 4 min and then 30 cycles at 94°C for 1 min, 50°C for 1 min, 72°C for 1.5 min and 1 cycle at 72°C for 4 min. The PCR product was digested with *Eco*R1 and *Bam*H1 and ligated into pEGFP-$N_2$ (Clonetech, Palo Alto, CA) cut with the same enzymes. The result was a cDNA which encodes for a fusion protein consisting of the ovine PGRMC1 and GFP. Identity of the fusion cDNA was confirmed by sequencing (UC Davis, Davis, CA).

Cellular localization of ovine PGRMC1: Chinese Hamster Ovary (CHO) and monkey kidney fibroblast (Cos7) cells were plated onto tissue culture dishes containing a #0 coverslip (MatTek Cultureware, Ashland, MA) in complete medium (CHO medium =

DMEM, supplemented with 10% FBS, non-essential amino acids (8.9 mg/L L-Alanine, 15 mg/L L-Asparagine, 13.3 mg/L L-Aspartic acid, 14.7 mg/L L-Glutamic acid, 7.5 mg/L Glycine, 11.5 mg/L L-Proline and 10.5 mg/L L-Serine) and penicillin-streptomycin (100 I.U. penicillin and 100 μg/mL streptomycin)); (Cos7 medium = DMEM supplemented with 10% FBS and penicillin-streptomycin (100 I.U. penicillin and 100 μg/mL streptomycin)). The following day cells were transfected with the vector (pEGFP-$N_2$) encoding PGRMC1-GFP using the Polyfect transfection procedure (QIAGEN, Valencia, CA). At 24-48 h post-transfection, cells were treated with Alexa 594 concanavalin A (10 μg/mL) (Molecular Probes Inc., Eugene, OR) in PBS, which specifically stains the plasma membrane. Cells were fixed with chilled 4% paraformaldehyde and images acquired using a confocal laser scanning microscope (LSM 510 Meta) at 488- and 543-nm lines of an argon ion laser to excite samples.

To determine if the ovine PGRMC1 was localized in the endoplasmic reticulum, CHO cells were co-transfected with the vector (pEGFP-$N_2$) encoding PGRMC1-GFP and a vector (pDsRed2-ER) (BD Biosciences, Palo Alto, CA), encoding a red fluorescent protein that is targeted to the endoplasmic reticulum. At 24 h post-transfection cells were fixed with chilled 4% paraformaldehyde and images acquired on a confocal laser scanning microscope as described previously.

Receptor Binding Assays. A crude membrane fraction was prepared from CHO cells and CHO cells transfected with cDNA for ovine PGRMC1 as described (Falkenstein, Heck et al. 1999). CHO cells were cultured in DMEM, supplemented with 10% FBS, non-essential amino acids (8.9 mg/L L-Alanine, 15 mg/L L-Asparagine, 13.3

mg/L L-Aspartic acid, 14.7 mg/L L-Glutamic acid, 7.5 mg/L Glycine, 11.5 mg/L L-Proline and 10.5 mg/L L-Serine) and penicillin-streptomycin (100 I.U. penicillin and 100 μg/mL streptomycin) and transfected with a vector (pcDNA3.1+) encoding ovine PGRMC1 using the Polyfect procedure (QIAGEN, Valencia, CA). At 48h post-transfection, transfected and non-transfected CHO cells were washed with cold PBS and removed from tissue culture plates using a cell scraper and cold PBS, and concentrated by centrifugation. Cells were washed again with cold PBS, counted using a hemacytometer and lysed by pushing through a 27-gauge needle 10 times in homogenization buffer (250 mM sucrose, 50 mM Tris, pH 7.5; plus protease inhibitors). The homogenates were centrifuged at 1000 x g for 5 min followed by 10 min at 2000 x g. The supernatants were collected and spun at 100,000 x g at 4°C for 1h. Next, supernatants were removed and pellets containing the membrane fractions were resuspended in buffer B (50 mM Tris, 1 mM $CaCl_2$, 1mM $MgCl_2$, 5 mM KCl; pH 6.0). Binding assays for P4 were performed as described (Meyer, Schmid et al. 1996) with slight modifications. Duplicate aliquots of membrane fractions from CHO cells transfected with PGRMC1 and from nontransfected cells were incubated in buffer B (total volume 0.3 mL) containing 20 nM $^3$H-P4. Nonspecific binding was determined in the presence of a 1000-fold excess of nonradioactive P4. After incubation for 1 h at room temperature the bound and free ligand were separated by the addition of 0.8 mL ice cold dextran-coated charcoal (0.3 g defined charcoal and 0.03 g Dextran [Sigma, St Louis, MO] in 100 mL of buffer B) and incubation on ice for 10 min. After centrifugation at 1100 x g for 15 min at 4°C, 0.9 mL supernatants were carefully removed, mixed with 5

mL of ScintiSafe scintillation cocktail (Fisher Chemical, Fair Lawn, NJ) and radioactivity quantified in a Beckman scintillation spectrometer. A crude membrane fraction from CHO cells stably expressing the ovine mPR was included as a positive control. Statistical analysis was performed using the Newman-Keuls Multiple Comparison Test in Prism (Version 4a, from GraphPad Software, Inc.) and significance was taken as a value of $P < 0.05$.

Measurement of Intracellular $Ca^{2+}$. Intracellular $Ca^{2+}$ was measured as described (Shlykov and Sanborn 2004). Briefly, CHO cells were plated in complete medium onto tissue culture dishes containing a #0 coverslip and the following day transfected with a mammalian expression vector encoding the ovine PGRMC1 (PGRMC1 in pcDNA3.1+) or control vector (pcDNA3.1+). At 24-48 h post-transfection, cells were loaded with Fura-2-AM (5 μM) (Molecular Probes, Eugene, OR) at room temperature for 30-35 min in fluorescence buffer (145 mM NaCl, 5mM KCl, 1 mM $Na_2HPO_4$, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 5 mM glucose, pH 7.4). After loading, cells were washed twice with fluorescence buffer and used after 35-45 min. Immediately prior to measuring $Ca^{2+}$, fluorescence buffer was removed and replaced with $Ca^{2+}$-free fluorescence buffer supplemented with EGTA (100 μM). Progesterone (1-100 nM) was added to cells and changes in free intracellular $[Ca^{2+}]_i$ in individual cells was measured at 340 and 380 nm excitation and 510 nm emission wavelengths with an InCyt2 imaging system (Intracellular Imaging Inc., Cincinnati, OH).

Results: A cDNA product of approximately 320 base pairs was amplified from ovine genomic DNA by PCR. Sequence analysis of the cDNA sample revealed a high homology to the reported *bos tarus* membrane steroid binding protein (Accession # AF254804) and the pig and human PGRMC1 (Accession # X99714 and NM_006667, respectively). Little to no homology existed between the PCR product for the ovine PGRMC1 and the sheep nuclear PR-A or –B at either the nucleotide or amino acid level.

Approximately 35 positive plaques were observed from the primary screen of the ovine CL d-10 cDNA library using the radiolabeled ovine PGRMC1 PCR product. Each clone was subjected to two additional rounds of screening before excision of pBluescript SK plasmid. A cDNA product of approximately 2000 base pairs was isolated from the ovine CL d-10 cDNA library. Sequence analysis of the cDNA sample confirmed the presence of an open reading frame, which encodes a protein with 194 amino acid residues (Fig 2.1). The ovine PGRMC1 contains a single 28 amino acid transmembrane domain at the N-terminus based on hydrophobicity analysis. The ovine PGRMC1 is 97%, 93% and 92% homologous to the Bos taurus PGRMC1 (Accession #XM_874120), Sus scrofa PGRMC1 (Accession #NM_213911) and Homo sapiens PGRMC1 (Accession #BC034238) at the nucleotide level, respectively.

Imaging experiments employing confocal fluorescence microscopy revealed that concanavalin A (ConA) specifically stained the plasma membrane red. Despite the single transmembrane domain (based on hydrophobicity analysis) for the ovine PGRMC1, no colocalization was observed between PGRMC1-GFP and concanavalin A in CHO (Fig 2.2) or Cos7 cells expressing PGRMC1-GFP (Fig 2.3). Expression of PGRMC1-GFP

appeared to be perinuclear, possibly in the endoplasmic reticulum. Since PGRMC1 was isolated from an ovine cDNA library prepared from luteal RNA, expression patterns of the PGRMC1-GFP fusion protein in isolated luteal cells was examined next. Similar localization of PGRMC1-GFP was observed in small and large ovine luteal cells with possible colocalization with ConA (Fig 2.4). To determine if the ovine PGRMC1-GFP was localized in the endoplasmic reticulum, studies were performed in CHO cells co-transfected with PGRMC1-GFP and pDsRed2-ER, which encodes for a red fluorescent protein that is targeted to the endoplasmic reticulum. Expression of PGRMC1-GFP yielded complete co-localization with the ER marker as presented in Fig 2.5.

To determine if P4 binds to the ovine PGRMC1, crude membrane fractions from CHO cells transfected with cDNA encoding PGRMC1 were incubated with $^{3}$H-P4. As shown in Fig 2.6, there were no differences between total binding and nonspecific binding in membrane fractions from transfected or nontransfected CHO cells. Only in the crude membrane fractions from CHO cells expressing the ovine mPR was P4 able to displace the binding of $^{3}$H-P4. Despite the lack of binding for P4 to the ovine PGRMC1, given the localization of PGRMC1 in the endoplasmic reticulum and the report that PGRMC1 was possibly involved in $Ca^{2+}$ influx (Falkenstein, Heck et al. 1999), we wanted to determine if treatment with P4 would cause an increase in intracellular $Ca^{2+}$ in cells expressing ovine PGRMC1. Treatment of CHO cells expressing PGRMC1 with P4 however, did not induce a rise in intracellular $Ca^{2+}$ (Fig 2.7).

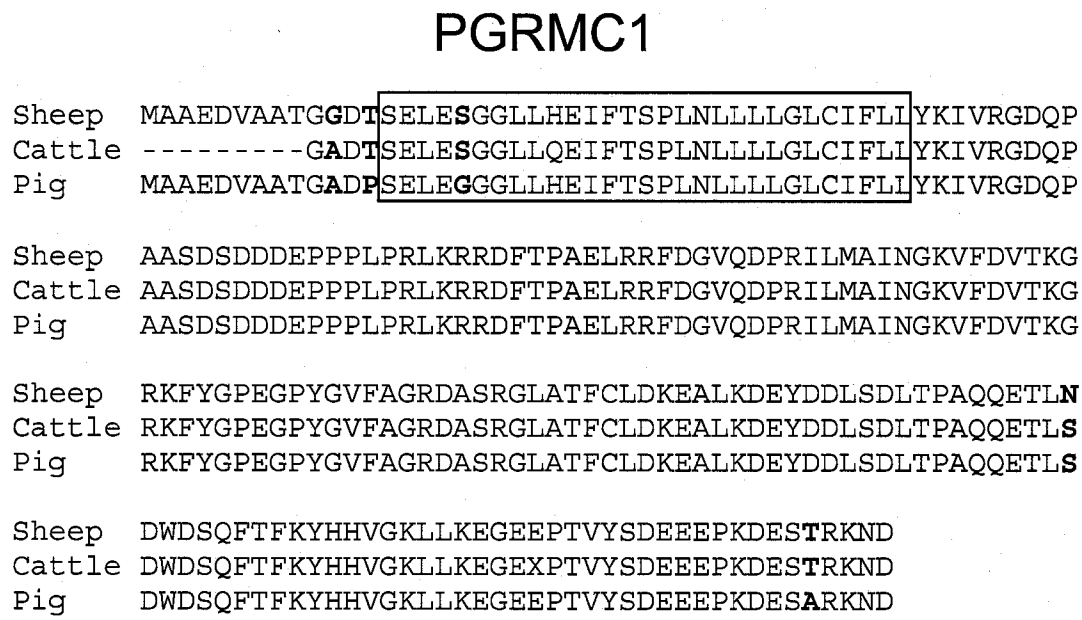


Figure 2.1. Amino acid composition of sheep, cattle, and pig progesterone receptor membrane component 1 (PGRMC1). The predicted transmembrane domain is indicated by the amino acids in the black box. Amino acids in bold type represent differences between the three species.

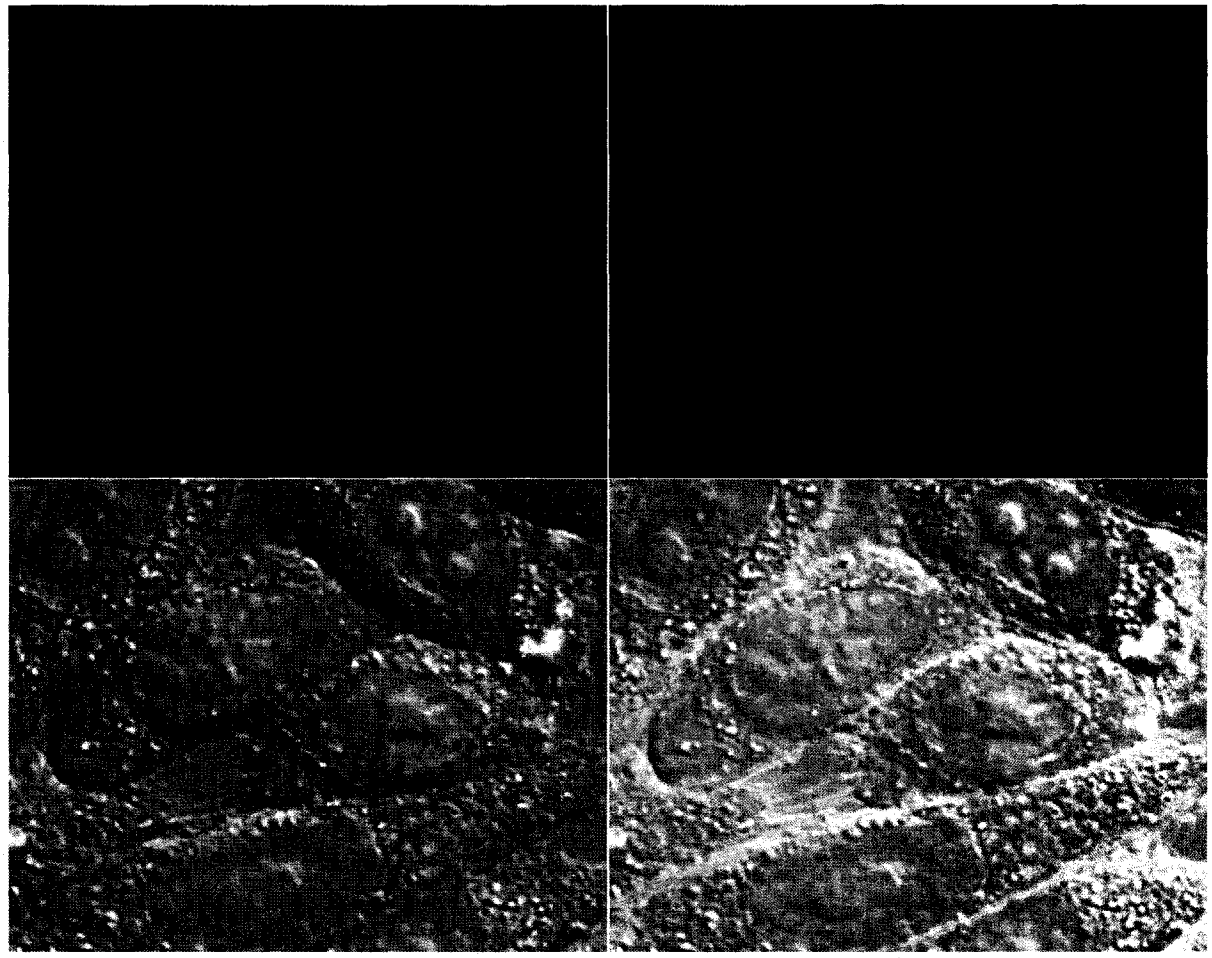

Figure 2.2. Confocal images of PGRMC1-GFP localization relative to plasma membrane. The top left panel shows a representative confocal image (63 X magnification) of CHO cells expressing PGRMC1-GFP. The plasma membrane was identified by Alexa 594 concanavalin A staining as shown in red in the top right panel. The bottom left panel is the Differential Interference Contrast image and a merged view of all images is presented in the bottom right panel. Co-localization of the proteins would appear as yellow.

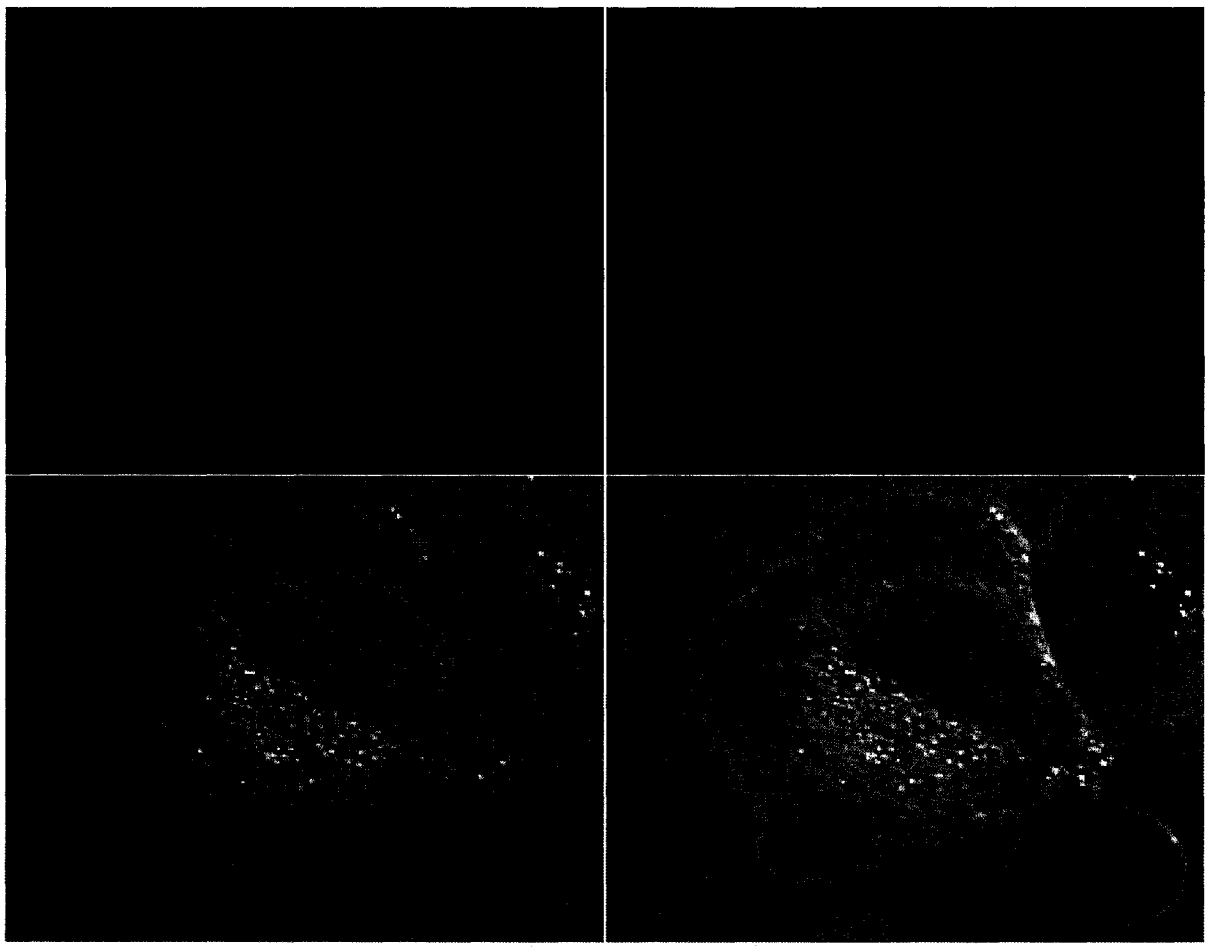

Figure 2.3. Confocal images of PGRMC1-GFP localization relative to plasma membrane. The top left panel shows a representative confocal image (63 X magnification) of Cos-7 cells expressing PGRMC1-GFP. The plasma membrane was identified by Alexa 594 concanavalin A staining as shown in red in the top right panel. The bottom left panel is the Differential Interference Contrast image and a merged view of all images is presented in the bottom right panel. Co-localization of the proteins would appear as yellow.

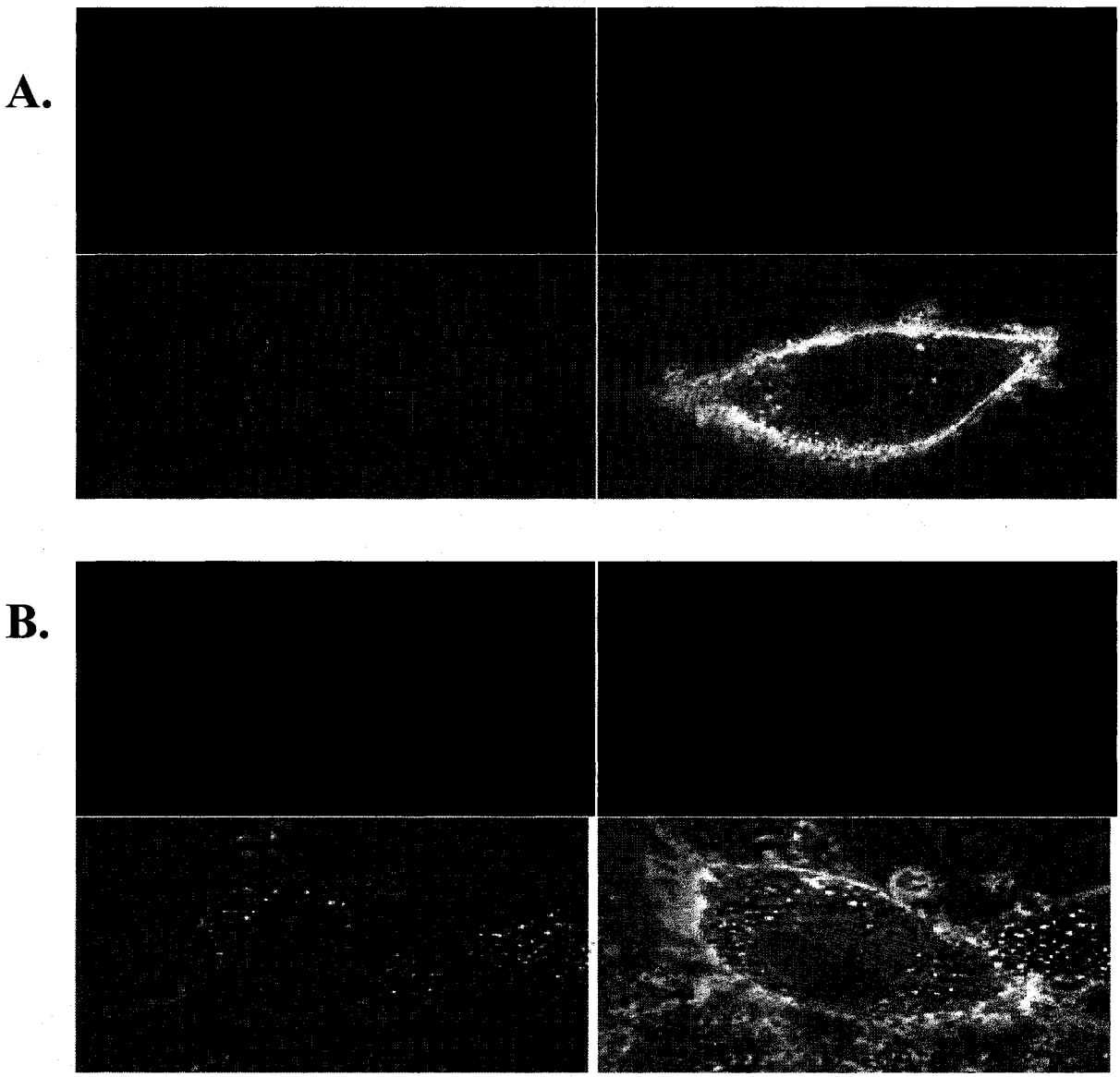


Figure 2.4. Confocal images of PGRMC1-GFP localization relative to plasma membrane in ovine luteal cells. A, Top left panel shows confocal image (63 X magnification) of small luteal cells expressing PGRMC1-GFP with the plasma membrane identified by Alexa 594 concanavalin A staining as shown in the top right panel. The bottom left panel is the Differential Interference Contrast image and a merged view of all images is presented in the bottom right panel. B, large luteal cells expressing PGRMC1-GFP. Co-localization of the proteins appears as yellow.

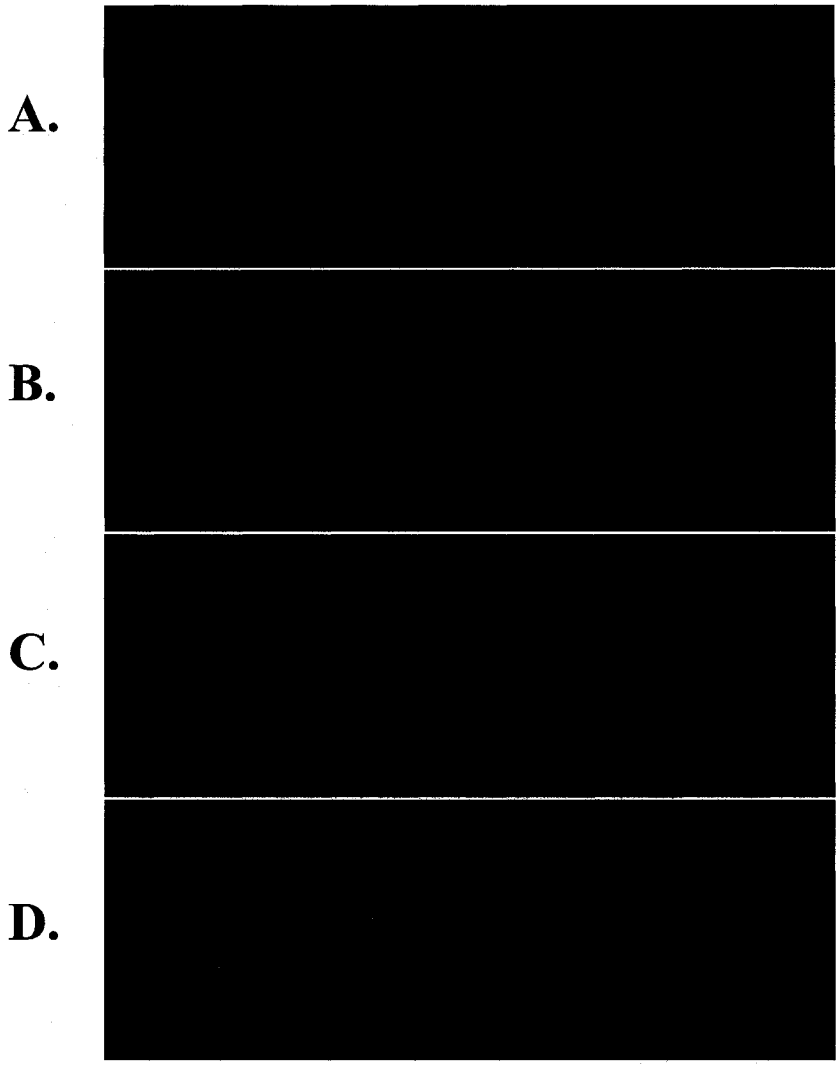


Figure 2.5. Localization of PGRMC1-GFP in the endoplasmic reticulum in CHO cells cotransfected with PGRMC1-GFP and pDsRed2-ER, an endoplasmic reticulum targeted red fluorescent protein. Confocal images (63 X magnification) were acquired 24-48 h post transfection. Panel A. displays expression of PGRMC1-GFP, panel B. is the Differential Interference Contrast image and panel C. is the expression of pDsRed2-ER. Panel D. is a merged view of both proteins. Co-localization of proteins is depicted in yellow.

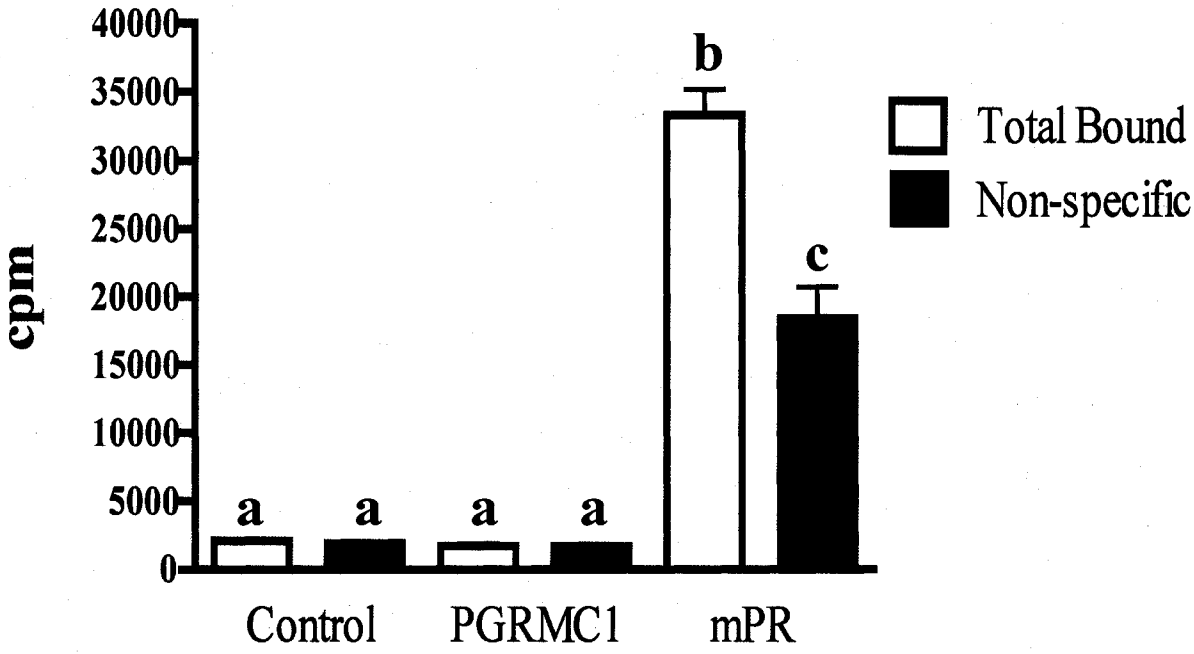


Fig 2.6 Binding of $^{3}$H-progesterone is not increased in crude membrane fractions from CHO cells expressing the ovine PGRMC1. A crude membrane fraction from CHO cells expressing the ovine mPR was included as a positive control. The control values represent crude membrane fractions from CHO cells. Displacement of $^{3}$H-progesterone was measured in the presence of 1000-fold excess nonradioactive progesterone (non-specific). Values are means ± S.E. (n = 3). Significant differences ($P < 0.05$) between groups are indicated by different letters above the bars. Open bars represent total binding and the black bars represent non-specifc binding in the presence of excess progesterone.

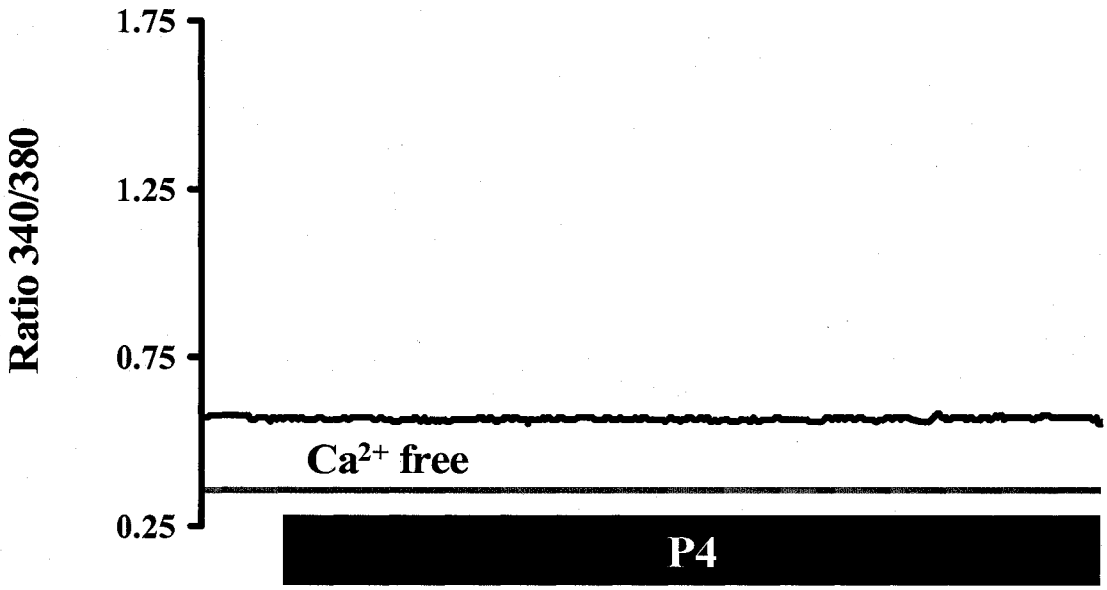


Figure 2.7. Effects of progesterone (P4) (1 nM) on $[Ca^{2+}]_i$ in CHO cells expressing ovine PGRMC1. The tracing is a representative from a minimum of three experiments in $Ca^{2+}$-free medium.

Discussion: Isolation, identification and cellular localization of an ovine membrane associated protein referred to as PGRMC1 is described in the current chapter. Specific binding of P4 has been demonstrated in CL membrane fractions from a variety of mammalian species (Bramley and Menzies 1988; Bramley and Menzies 1988; Rae, Menzies et al. 1998), including sheep (Bramley and Menzies 1994), yet the protein responsible is not known. Experiments were performed to determine if PGRMC1, a known membrane associated protein that allegedly binds P4, was expressed in ovine corpora lutea. PCR was performed using ovine genomic DNA and a product of approximately 320 base pairs was amplified. Subsequent sequence analysis of the PCR product revealed high homology to the reported *bos tarus* membrane steroid binding protein (Accession # AF254804) and the pig and human PGRMC1 (Accession # X99714 and NM_006667, respectively) but there was no homology to the sheep nuclear PR-A or –B. From the initial PCR experiment, I knew the gene encoding PGRMC1 was present in the sheep; however, whether PGRMC1 was expressed in ovine CL was not known. To address this issue, a cDNA expression library generated from ovine CL at d-10 of the cycle was screened. For these experiments the isolated PCR product was radiolabeled with $^{32}P$ and subsequently used to screen the cDNA expression library. Results from these experiments confirmed the presence of a full length cDNA clone encoding PGRMC1 in ovine CL. The ovine PGRMC1 is 97%, 93% and 92% homologous to the Bos taurus PGRMC1 (Accession #XM_874120), Sus scrofa PGRMC1 (Accession

#NM_213911) and Homo sapiens PGRMC1 (Accession #BC034238) at the nucleotide level, respectively. Similar to PGRMC1 reported in other mammals, the ovine PGRMC1 consists of 194 amino acids and contains a single predicted transmembrane domain at the N-terminus. In support of PGRMC1 expression in the CL, similar results have been demonstrated in CL from rat (Cai and Stocco 2005; Peluso, Pappalardo et al. 2006) and human (Sasson, Rimon et al. 2004). PGRMC1 was originally isolated from porcine liver and has also been found in numerous other tissues including kidney, lung, cerebellum, spleen, heart (Falkenstein, Meyer et al. 1996) and sperm (Losel, Dorn-Beineke et al. 2004). Also, mRNA encoding PGRMC1 exists in preovulatory mouse follicles (McRae, Johnston et al. 2005), porcine granulosa cells (Jiang, Whitworth et al. 2004), human sperm (Losel, Breiter et al. 2005), human granulosa cells maintained in culture (Sasson, Rimon et al. 2004) and bovine lens epithelial cells (Zhu, Sexton et al. 2001). Given the ubiquitous nature of PGRMC1, it would not be surprising to find PGRMC1 expressed in many other ovine tissues.

Based on the transmembrane domain present in the N-terminus of the ovine PGRMC1, it seemed plausible PGRMC1 might reside in the plasma membrane. However, the ovine PGRMC1 does not appear to localize to the plasma membrane based on confocal microscopy experiments with the PGRMC1-GFP fusion protein. Initial studies employing the PGRMC1-GFP construct displayed distinct fluorescence around the nucleus, possibly in an intracellular tubular network or the cytosol. Experiments were conducted in two different cell lines expressing the PGRMC1-GFP fusion protein to confirm that the localization of PGRMC1-GFP was not due to cell lineage. Each time

subcellular localization appeared to be in an intracellular membrane or the cytosol. Since the ovine PGRMC1 was isolated from the CL, the cellular localization of PGRMC1-GFP was determined in transfected luteal cells. Once again, there was perinuclear expression of PGRMC1-GFP with possible colocalization in the plasma membrane.

Data from confocal microscopy experiments utilizing CHO cells expressing both PGRMC1-GFP and ER-dsRed provided further support for localization of PGRMC1 in the endoplasmic reticulum; complete co-localization was observed between PGRMC1-GFP and the endoplasmic reticulum marker. In support of these observations, PGRMC1 was found localized in the endoplasmic reticulum and/or golgi apparatus using a peptide specific antibody with porcine hepatocytes (Falkenstein, Schmieding et al. 1998). These researchers also demonstrated that binding of $^{3}$H-P4 and glucose-6-phosphatase activity, a marker enzyme for endoplasmic reticulum, were closely correlated in subcellular fractions of porcine liver cells. In contrast, a recent study using rat luteal cells, demonstrated that PGRMC1 was distributed throughout the cell (Peluso, Pappalardo et al. 2006) with localization found in both the plasma membrane and cytoplasm. Since our studies utilized the ovine PGRMC1, there are possible species differences which may account for the dissimilarity noted in cellular localization. On the other hand, in rat luteal cells the cellular localization of PGRMC1 for the most part mimics the localization of PAIRBP1, a protein which localizes to the plasma membrane and interacts with PGRMC1 (Peluso, Pappalardo et al. 2006). It seems quite probable that PAIRBP1 is also expressed in ovine luteal cells, which could account for the slight colocalization of PGRMC1-GFP with ConA in small and large cells (Fig 2.4). It is important to

underscore that the data presented in this chapter do not eliminate the possibility of plasma membrane expression but certainly suggest prominent localization to the endoplasmic reticulum.

PGRMC1 was isolated first from porcine liver and these researchers also reported binding of $^{3}$H-P4 in crude membrane fractions from liver (Meyer, Schmid et al. 1996) which they correlated with the porcine PGRMC1. This same laboratory tried to further evaluate the binding of P4 for the porcine PGRMC1 by transfecting CHO cells with a vector encoding the porcine PGRMC1 (Falkenstein, Heck et al. 1999). These researchers reported increased binding of P4 in microsomal fractions from transfected CHO cells; however the binding is poor at best with an apparent binding capacity of 1.16 pmol/mg protein in CHO cells expressing porcine PGRMC1 compared to 0.69 pmol/mg protein in CHO cells transfected with control vector. Further, the $IC_{50}$ for P4 was 0.5 μM (Falkenstein, Heck et al. 1999). To investigate the possible binding of P4 to the ovine PGRMC1, experiments were conducted in CHO cells expressing ovine PGRMC1 similar to the methods reported for the porcine PGRMC1 (Falkenstein, Heck et al. 1999). Contrary to the porcine PGRMC1, there was no difference between total binding of $^{3}$H-P4 and nonspecific binding (1000-fold excess of P4) in membrane fractions from CHO cells expressing ovine PGRMC1 (Fig 2.6). Therefore, the ovine PGRMC1 does not appear to bind P4. Additionally, our data do not support the notion that PGRMC1 is involved in mobilization of intracellular $Ca^{2+}$ since addition of P4 did not induce any changes in concentrations of intracellular $Ca^{2+}$ in CHO cells expressing ovine PGRMC1 (Fig 2.7). These results are also in contrast to the porcine PGRMC1 (Falkenstein, Heck

et al. 1999). Results from the porcine PGRMC1 paper however were based on indirect evidence in which treatment with an antibody specific to the porcine PGRMC1 reduced the P4-induced increase in $Ca^{2+}$ in human sperm prepared under capacitating conditions. Interestingly, the monoclonal antibody (C-262) which binds to nPRs also inhibits the P4-induced $Ca^{2+}$ influx and the acrosome reaction in human sperm (Sabeur, Edwards et al. 1996). Since the PGRMC1 displays extremely little homology to the nPRs, it seems doubtful the C-262 antibody would bind PGRMC1 and inhibit the increase in $Ca^{2+}$. Thus, it is unlikely that PGRMC1 is solely responsible for the increase in $Ca^{2+}$ in sperm treated with P4. These data do not rule out the possibility that the PGRMC1 may be involved in the $Ca^{2+}$ increase in sperm, but strongly support the involvement of other proteins.

## CHAPTER III.

## CLONING AND CHARACTERIZATION OF AN OVINE INTRACELLULAR TRANSMEMBRANE RECEPTOR FOR PROGESTERONE

Introduction: As discussed in Chapter 2, a cDNA clone was isolated from an ovine CL d-10 cDNA library and encodes a 194 amino acid protein referred to as PGRMC1. The ovine PGRMC1 also contains a single transmembrane domain similar to the porcine PGRMC1 (Falkenstein, Meyer et al. 1996; Meyer, Schmid et al. 1996). One objective of the research conducted for this dissertation was to isolate a mPR from sheep, distinct from the nPR that displayed plasma membrane localization. Studies investigating the cellular localization of the ovine PGRMC1 demonstrated localization predominately in the endoplasmic reticulum and not the plasma membrane. While investigating the cellular localization of the sheep PGRMC1, an independent study was published describing the cloning and characterization of a novel mPR isolated from a seatrout ovarian cDNA library that appeared to be a GPCR (Zhu, Rice et al. 2003). These researchers also suggested the seatrout mPR was a plasma membrane receptor. Recombinant mPR expressed in *Escherichia coli* binds specifically to P4 and 17-hydroxyprogesterone in vitro with a relatively high affinity ($k_d$ = 30 nM). Further, the deduced amino acid sequences from the sea trout mPR cDNA predicted seven transmembrane domains, a characteristic of GPCRs, but not nPRs.

To date three isoforms of this mPR have been identified (i.e., mPR$\alpha$, mPR$\beta$, and mPR$\gamma$) (Zhu, Bond et al. 2003). Expression of the $\alpha$ isoform is mainly in reproductive

tissues, whereas the $\beta$ isoform is expressed mainly in the brain. The $\gamma$ isoform is expressed primarily in kidney, lung and colon. Homologous transcripts for these isoforms were also confirmed in mammals, specifically human, mouse, pig (Zhu, Bond et al. 2003) and rat (Cai and Stocco 2005). My working hypothesis at the time was that the nongenomic effects of P4 reported in sheep coupled with the specific binding of P4 in membrane fractions from ovine CL was occurring through a unique PR at the plasma membrane. Given the unique structure of the seatrout mPR (i.e., GPCR) and data demonstrating rapid induction of second messenger pathways (Zhu, Rice et al. 2003) upon ligand activation of mPR, I wanted to determine if this mPR was also present in the sheep. Consequently, experiments were designed to isolate the mPR$\alpha$ isoform from sheep since this isoform was predominately found in reproductive tissues (Zhu, Bond et al. 2003) and to determine the tissue distribution and cellular localization of the ovine mPR. Next, studies were completed to investigate the possible changes in expression of the ovine mPR mRNA throughout the estrous cycle.

## Materials and Methods

Isolation and cloning of ovine mPR: PCR primers for isolation of the putative ovine mPR were designed from homologous sequences between the pig and human mPR$\alpha$ sequences (GenBank Accession numbers AF313616 and AF313620, respectively) as reported by Zhu et al., (Zhu, Bond et al. 2003). Sense ovine mPR$\alpha$ primer was 5' TCCCTGCCCCACCCACAGCCATG 3' and the antisense ovine mPR$\alpha$ primer was 5' CAGACACAAACAACTTTACCAGG 3'. Ovine genomic DNA served as template for

PCR, because the coding region for the human and pig mPR$\alpha$ lacks introns. Amplified DNA was analyzed on a 1% agarose gel and visualized via UV light illumination. The PCR product was excised and purified from the agarose using a QIAEX II Gel Extraction Kit (QIAGEN, Valencia, CA) and subsequently ligated into pGEMT-Easy vector (Promega, Madison, WI) per the manufacture's instructions. Identity of the cDNA sample was confirmed by sequencing (UC Davis, Davis, CA).

RNA preparation and tissue distribution of ovine mPR: All procedures involving animals were approved by the Colorado State University Animal Care and Use Committee. Numerous tissues were excised from sheep that had been euthanized with an overdose of sodium pentobarbitol and snap frozen in liquid nitrogen until isolation of RNA. Total RNA was isolated using TRI-Reagent per manufacture's instructions (Sigma, St. Louis, MO) and each RNA sample was then subjected to the TURBO DNA-free protocol (Ambion, Austin, TX) to ensure absence of DNA contamination. Concentration of RNA was determined by spectrophotometry, and integrity of RNA verified by gel electrophoresis in 1% agarose in the presence of ethidium bromide followed by visualization under UV light. To determine which tissues express mRNA for the ovine mPR, RT-PCR was performed via the SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen, Carlsbad, CA) using 1$\mu$g total cellular RNA per sample. Primers were designed based on homology between pig and sheep mPR$\alpha$ sequences and pig ovarian RNA served as a positive control for RT-PCR. Sense primer was 5' ACCTCCTGCAGGCCAAGTCTG 3' and the antisense primer was 5' TCCTGGCAAGTGCGGCCCAG 3'. Absence of genomic DNA in RNA preparations

was verified by omitting the RT/Platinum Taq mix and substituting Taq DNA polymerase in the reaction for each RNA sample tested.

Isolation of ovine pituitary and luteal cells: Anterior pituitary glands were collected following anesthesia of ewes with sodium pentobarbitol and exsanguination. Tissues were removed and immediately placed in ice cold dissociation medium consisting of 137 mM NaCl; 5 mM KCL; 25 mM n-2-hydroxyethyl piperazine ethane sulfonic acid (HEPES; United States Biochemical Corp., Cleveland, OH); pH 7.3, plus an enzymatic cocktail containing 1.0 mg/mL collagenase (type II), 1.0 mg/mL hyaluronidase (type V), and 0.02 mg/mL deoxyribonuclease. Enzymes were freshly prepared immediately prior to dissociation. Pituitary culture medium consisted of Dulbecco's Modified Eagle's Medium (DME; Gibco, Lab.) supplemented with 10 % OVX ewe serum, streptomycin sulfate (500 mg/mL), potassium penicillin G (313 mg/mL) and 2.2 g/L $NaHCO_3$. Dissociation and culture medium, as well as the enzymatic cocktail were sterilized by filtration through 0.2 μm Millipore membranes (type GS, Millipore Corp.). Pituitary tissue was dispersed as described by Adams et al. (Adams, Wagner et al. 1979) with the omission of trypsin digestion. Briefly, tissue was sectioned into 0.5-mm-thick slices using a Stadie-Riggs tissue slicer and washed 5 times with dissociation medium without enzymes. Tissue was incubated in dissociation medium containing the enzymatic cocktail at 37° C in a Dubnoff metabolic shaker for 90 min and every 30 min the cell suspension was passed through a Pasteur pipette. After dissociation, the cell suspension was washed (400 x g; 4 min) 5 times with dissociation medium without enzymes, re-

suspended in DMEM and plated at 2 x $10^6$ cells per well in tissue culture dishes containing a #0 coverslip (MatTek Cultureware, Ashland, MA).

Corpora lutea were collected surgically from superovulated western range ewes on day 10 post ovulation (Sheridan, Phillips et al. 1975), decapsulated and dissociated into single cells using collagenase (Ahmed and Niswender 1981). Single cell suspensions were separated into partially purified small and large cell fractions by elutriation (Fitz, Mayan et al. 1982). Cells were cultured in DMEM, supplemented with 10% FBS and penicillin-streptomycin (100 I.U. penicillin and 100 μg/mL streptomycin).

Relative expression of mRNA for ovine mPR throughout the estrous cycle: Ewes were exposed to a vasectomized ram to determine when they came into estrus. On day 13-14 of the cycle, ewes were administered Lutalyse (7.5 mg I.M.) two times, 4 h apart. Before the first dose of Lutalyse was given, jugular blood samples were taken from each ewe for analysis of serum concentrations of P4. Jugular blood samples were also taken daily from each ewe until time of euthanization. Blood was allowed to clot for 1 h at room temperature and serum was collected by centrifugation. Serum was stored at -20°C until analyzed for concentrations of P4. Estrus was detected using a vasectomized ram and ewes were assigned to various groups depending on when they came into heat. Groups consisted of 3 and 24 h post estrus and days 4, 10, and 15 of the estrous cycle. At the appropriate time of the cycle, tissues were excised from sheep following anesthesia of ewes with sodium pentobarbitol and exsanguination, and snap frozen in liquid nitrogen until isolation of RNA. Total RNA was extracted using the RNeasy Midi Kit (QIAGEN, Valencia, CA) according to manufacturer's instructions. To ensure RNA samples were

not contaminated with genomic DNA, each sample was subjected to RNase-Free DNase treatment (QIAGEN, Valencia, CA). Concentration of RNA was determined by spectrophotometry, and integrity of RNA verified by gel electrophoresis in 1% agarose in the presence of ethidium bromide followed by visualization under UV light. A set concentration of RNA (1 μg) was reverse-transcribed into cDNA, by using the iScript cDNA Synthesis Kit employing the reverse transcriptase RNAse H+ (Bio-Rad Laboratories, Hercules, CA) per manufacturer's instructions. The RT products were diluted to a final volume of 100 μL. Quantitative PCR was performed on a Bio-Rad iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Real-time quantitative PCR was performed in single wells of a 96-well plate (Bio-Rad Laboratories, Hercules, CA) using components of the iQ SYBR Green supermix (2X), 0.5 μM forward and reverse specific primer, and 10μL of the RT product. As a negative control for all the reactions, preparations lacking RNA or reverse transcriptase were used in place of the cDNA. The following primers were used for PCR amplification: Ovine mPRα, TTGTGGGCACCGTGGACTTC and GCTAAGGCACTGAGGGAGAGG; Ovine β-actin TCTGGCACCACACCTTCTAC and GGTCATCTTCTCACGGTTGG. The following PCR thermocycling program was used: 95° C for 3 min, followed by 40 cycles consisting of 95°C for 10 sec and 55°C for 45 sec, followed by one cycle each of 95°C for 1 min, and 55°C for 1 min and a melt curve. Fluorescence was measured after each cycle and displayed graphically (iCycler iQ Real-Time Detection System Software, version 3.1; Bio-Rad). The software determined the cycle threshold (Ct) values for each sample. For analysis, quantitative amounts of ovine mPR were standardized against the

house-keeping gene β-actin. RNAs were assayed from three to five independent biological replicates. Statistical analysis was performed on the normalized data using the Newman-Keuls Multiple Comparison Test in Prism (Version 4a, from GraphPad Software, Inc.) and significance was taken as a value of $P < 0.05$. When a significant difference ($P < 0.05$) was noted, the RNA levels were reported as a fold change, using the "Delta-delta method" for comparing relative expression results in real-time PCR (Pfaffl 2001). Data are shown as the mean ± S.E. of each normalized measurement.

Radioimmunoassay for serum progesterone: The concentration of P4 in serum from the ewes was quantified by a double antibody radioimmunoassay (Niswender 1973). Triplicate standard curves were included in each assay and samples were analyzed in duplicate at 200 μL sample/tube.

Cellular localization of ovine mPR: Fusion proteins consisting of the putative ovine mPR fused to green fluorescent protein (GFP) or hemagluttinin (HA) were used for cellular localization. A C-terminal GFP fusion to the putative ovine mPR was generated by PCR using cDNA containing the full coding sequence of the mPR, with a gene-specific primer that inserted an *Eco*R1 site upstream of the transcription start site and a gene-specific primer that eliminated the stop codon and substituted a *Bam*H1 site at its 3'-end to create in-frame restriction sites. The PCR product was digested with *Eco*R1 and *Bam*H1 and ligated into pEGFP-$N_2$ (Clonetech, Palo Alto, CA) cut with the same enzymes. The result was a cDNA encoding for a fusion protein consisting of the putative ovine mPR and GFP. Identity of the fusion cDNA was confirmed by sequencing (UC Davis, Davis, CA). The HA-mPR fusion protein was generated by inserting the coding

sequence for the putative ovine mPR into the pKH3 vector. The result was a cDNA encoding for a fusion protein consisting of 3 HA peptides on the N-terminus of the putative ovine mPR. Identity of the fusion cDNA was confirmed by sequencing (UC Davis, Davis, CA). To determine if mPR is present in the cell membrane, Chinese Hamster Ovary (CHO) cells were plated onto tissue culture dishes containing a #0 coverslip (MatTek Cultureware, Ashland, MA) in complete medium (DMEM, supplemented with 10% FBS, non-essential amino acids (8.9 mg/L L-Alanine, 15 mg/L L-Asparagine, 13.3 mg/L L-Aspartic acid, 14.7 mg/L L-Glutamic acid, 7.5 mg/L Glycine, 11.5 mg/L L-Proline and 10.5 mg/L L-Serine) and penicillin-streptomycin (100 I.U. penicillin and 100 μg/mL streptomycin)). The following day cells were transfected with a vector (pEGFP-$N_2$) encoding mPR-GFP using the Polyfect transfection procedure (QIAGEN, Valencia, CA). Luteal and pituitary cells were treated identically, except transfection was accomplished with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). At 24-48 h post-transfection, cells were treated with Alexa 594 concanavalin A (Molecular Probes Inc., Eugene, OR) in PBS, which specifically stains the plasma membrane. Cells were fixed with chilled 4% paraformaldehyde and images acquired on a confocal laser scanning microscope (LSM 510 Meta) using 488- and 543-nm lines of an argon ion laser to excite samples.

For immunocytochemical experiments, CHO cells were plated as previously described and transfected with a vector (pKH3) encoding HA-mPR using the Polyfect transfection procedure (QIAGEN, Valencia, CA). At 24-48h post-transfection, cells were washed with cold PBS, fixed with chilled 4% paraformaldehyde, washed again with cold

PBS and permeabilized with PBS containing 0.3% Triton X-100 for 10 min at room temperature. Cells were again washed with cold PBS and incubated with PBS plus 3% BSA for 1 h at room temperature to minimize non-specific binding. Cells were incubated with rabbit polyclonal anti-HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:250 dilution in PBS plus 3% BSA overnight at 4°C. Then cells were washed with cold PBS plus 0.3% Triton X-100 and incubated with goat anti-rabbit Alexa 488 antibody (Molecular Probes Inc., Eugene, OR) at 1:200 dilution in PBS plus 3% BSA for 1 h at room temperature and protected from light. Cells were then washed with cold PBS and images acquired using confocal microscopy similar to mPR-GFP experiments. To determine if there was non-specific binding, background fluorescence was determined in cells transfected with HA-mPR and subjected to secondary antibody alone.

For Western blot analysis, Cos7 (monkey kidney fibroblast) cells grown in complete medium (DMEM, supplemented with 10% FBS and penicillin-streptomycin (100 I.U. penicillin and 100 μg/mL streptomycin)) were transfected with the vector (pKH3) encoding HA-mPR using the Polyfect procedure (QIAGEN, Valencia, CA). At 24-48h post-transfection, Cos7 cells transfected with HA-mPR and non-transfected cells were washed with cold PBS and cytosolic and membrane fractions were prepared as described previously (Soloff and Sweet 1982) with slight modifications. Briefly cells were removed from tissue culture plates using a cell scraper and homogenization buffer (100 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-HCL, 1mM EGTA, plus protease inhibitors; pH 7.2) and collected by centrifugation. Cells were homogenized and cellular debris

collected by centrifugation using a table top centrifuge at 5000 x g at room temperature for 15 min. Supernatants were collected and spun at 100,000 x g at 4°C for 1h. Supernatants were removed and kept separate while pellets containing the membrane fractions were resuspended in cold sample buffer (10 mM Tris-HCL, 250 mM sucrose, 1mM EGTA, plus protease inhibitors; pH 7.2). Cytosolic proteins were brought to a total volume of 1 mL with distilled water and 100 μL of 0.15% deoxycholate was added, mixed and incubated for 10 min at room temperature. Next, 100 μL of 72% TCA were added, mixed and incubated on ice for 20 min. Then samples were centrifuged for 15 min at 16,000 x g at room temperature and the pellet was washed 3 times with acetone to remove TCA. The pellet was air dried and then dissolved in cold sample buffer. Protein content was determined for each sample (cytosol and membrane) using the Coomassie Plus Protein Assay Kit (Pierce, Rockford, IL). Equal amounts of protein from each fraction were separated using denaturing PAGE followed by transfer to a nitrocellulose membrane for electroblotting. Samples were analyzed for HA-mPR by western analysis using a monoclonal anti-HA antibody (Roche Diagnostics, Indianapolis, IN) at 1:1000 dilution in 5% non-fat milk made in Tris-buffered saline plus Tween-20 (TBST). Proteins were detected using chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate; Pierce, Rockford, IL).

To determine if the ovine mPR was localized in the endoplasmic reticulum, CHO cells were co-transfected with the vector (pEGFP-$N_2$) encoding mPR-GFP and the vector pDsRed2-ER (BD Biosciences, Palo Alto, CA), encoding a red fluorescent protein that is targeted to the endoplasmic reticulum. At 24 h post-transfection cells were fixed with

chilled 4% paraformaldehyde and images acquired on a confocal laser scanning microscope as described previously.

Results: A cDNA product of approximately 1100 base pairs was amplified from ovine genomic DNA by PCR. Sequence analysis of the cDNA sample confirmed the presence of an open reading frame corresponding to 1052 base pairs on one exon, which encodes a protein with 350 amino acid residues (see footnote)[1]. The putative ovine mPR is 97%, 91% and 83% homologous at the amino acid level to the reported pig, human and rat mPR$\alpha$, respectively (Fig 3.1). Likewise, the putative ovine mPR contains seven transmembrane domains based on hydrophobicity analysis (DAS-domain prediction and TmPRED). Despite similarity between the putative ovine mPR and human and pig mPR$\alpha$, almost no homology exists between the putative ovine mPR and the sheep nuclear PR-A or –B at either the nucleotide or amino acid level.

RT-PCR was performed on DNase-treated total RNA isolated from ovine hypothalamus, pituitary, uterus, ovary, CL, heart, muscle, lung, liver, kidney, spleen, caruncle and cotyledon. Tissues which expressed the ovine mPR gene were hypothalamus, pituitary, uterus, ovary, and CL; no bands were detected in reactions performed with identical RNA samples using Taq DNA polymerase instead of RT/Platinum Taq, verifying the absence of genomic DNA contamination (Fig 3.2 A).

[1] This sequence data has been submitted to DDBJ/EMBL/GenBank databases under accession number DQ318952

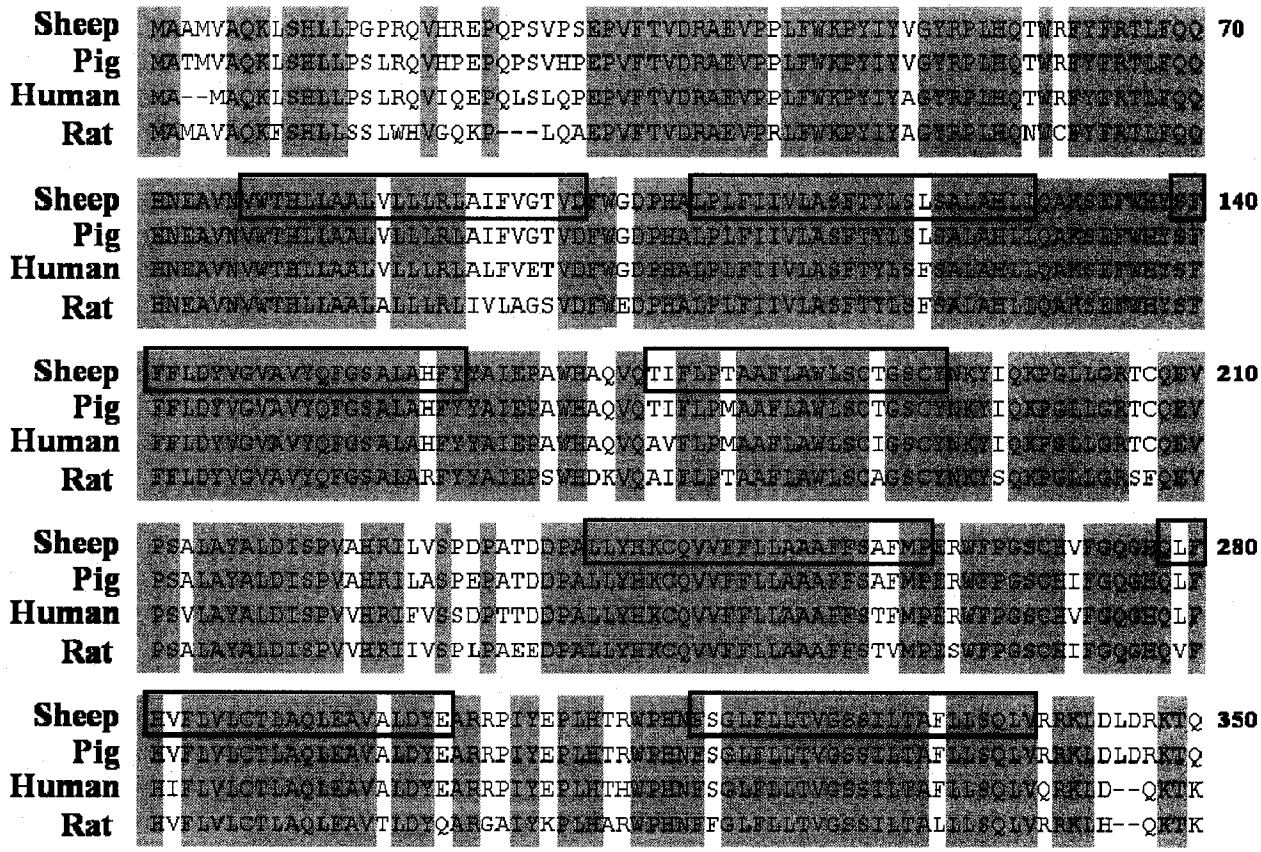


Figure 3.1. The deduced amino acid sequence of the sheep mPR$\alpha$ with similarities between sheep, pig, human and rat mPR$\alpha$ indicated by gray boxes and the seven transmembrane domains contained within the black-lined boxes. The sheep mPR$\alpha$ consists of 350 amino acids and is 97%, 91% and 83% homologous to the pig, human and rat mPR$\alpha$, respectively.

As the coding region for the ovine mPR consists of only one exon, it was critical to ensure no DNA contamination existed in the RNA samples, since this would invalidate the tissue distribution results. Figure 3.2 B displays tissues that tested negative for ovine mPR expression.

For the experiments investigating possible changes in expression of mPR throughout the ovine estrous cycle, jugular blood samples were collected and analyzed for serum concentrations of P4 to ensure ewes were at the appropriate time of their cycle. Specifically, ewes were euthanized and tissues collected at 3 and 24 h post estrus and then at days 4, 10, and 15 of the estrous cycle. As shown in Figure 3.3 all of the ewes appeared to be synchronized and displayed low concentrations of serum P4 at the beginning of their cycle which then elevated throughout and began to decline at the end of the cycle. No apparent differences were observed for mean relative expression of mRNA for mPR$\alpha$ in hypothalamus (Fig 3.4), ovary (Fig 3.5), or pituitary (Fig 3.6) throughout the estrous cycle. In the CL, the highest expression of mRNA for mPR$\alpha$ was observed on d 10 and was significantly greater ($P < 0.01$) compared to expression of mPR$\alpha$ on d 4, 3h and 24 h post estrus (Fig 3.7). Additionally, the expression of mPR$\alpha$ in the CL was significantly greater ($P < 0.05$) on d 15 compared to 3 h and 24 h post estrus (Fig 3.7). The mRNA for mPR$\alpha$ in the CL on d 10 displayed a 2.5, 3.9, and 4.5 fold higher expression compared to d 4, 3h, and 24 h post estrus, respectively. The expression on d 15 for mPR$\alpha$ in the CL was 2.2 and 2.6 fold higher expressed compared to 3 h and 24 h post estrus, resectively. The highest expression of mRNA for mPR$\alpha$ in the uterus was on d 4 and was significantly ($P < 0.01$) greater than mRNA at 3 h post estrus (Fig

3.8). The mRNA for mPR$\alpha$ in the uterus on d 4 displayed a 3.7 fold higher expression compared to 3h post estrus.

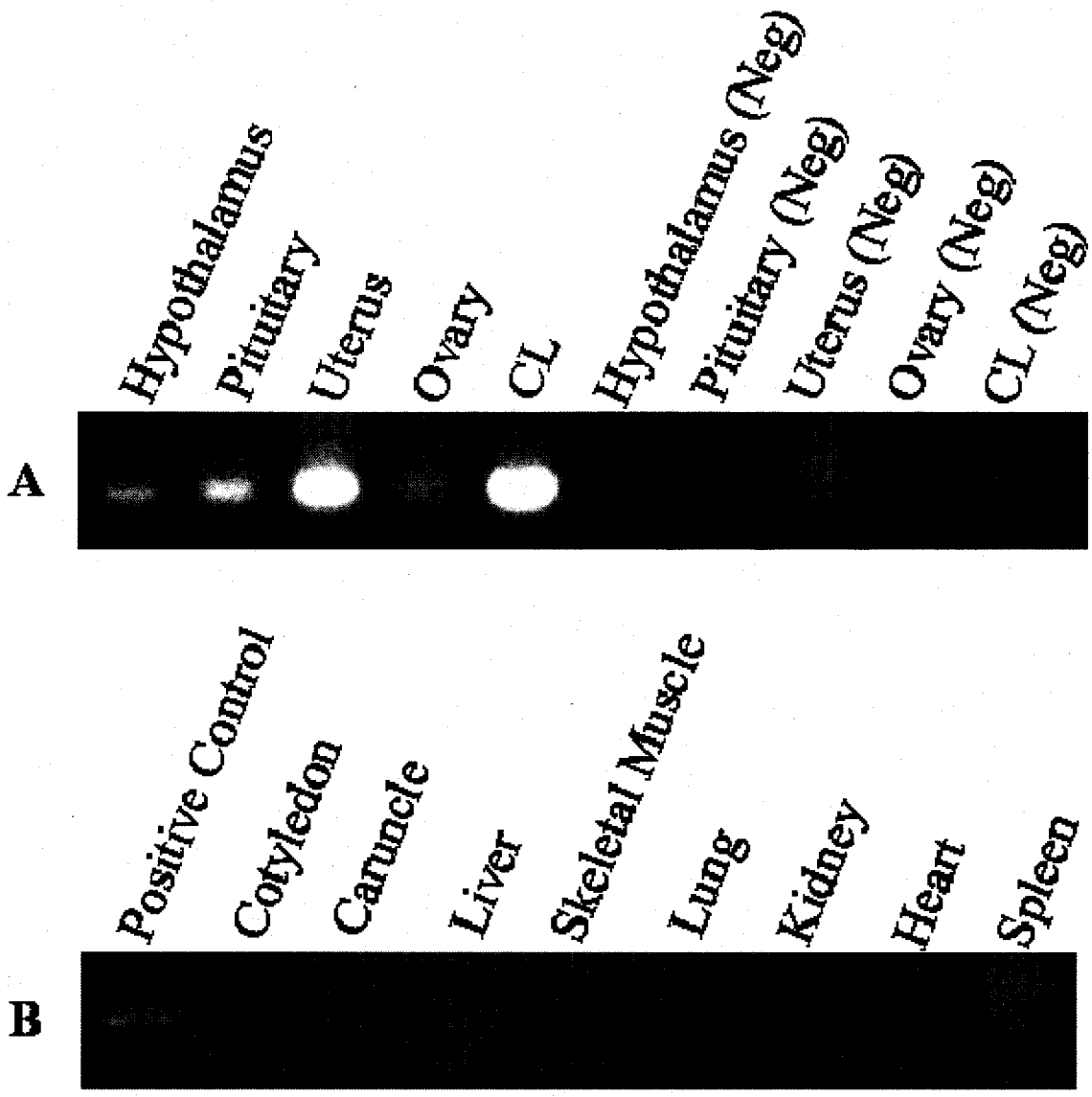


Figure 3.2. A, RT-PCR analysis showing mRNA expression of ovine mPRα in various sheep tissues. Absence of genomic DNA in RNA preparations was verified by omitting the RT/Platinum Taq mix and substituting Taq DNA polymerases in the reaction for each RNA sample tested and is represented as (Neg). B, RT-PCR analysis of ovine tissues tested not expressing mPRα. Pig ovarian RNA served as a positive control.

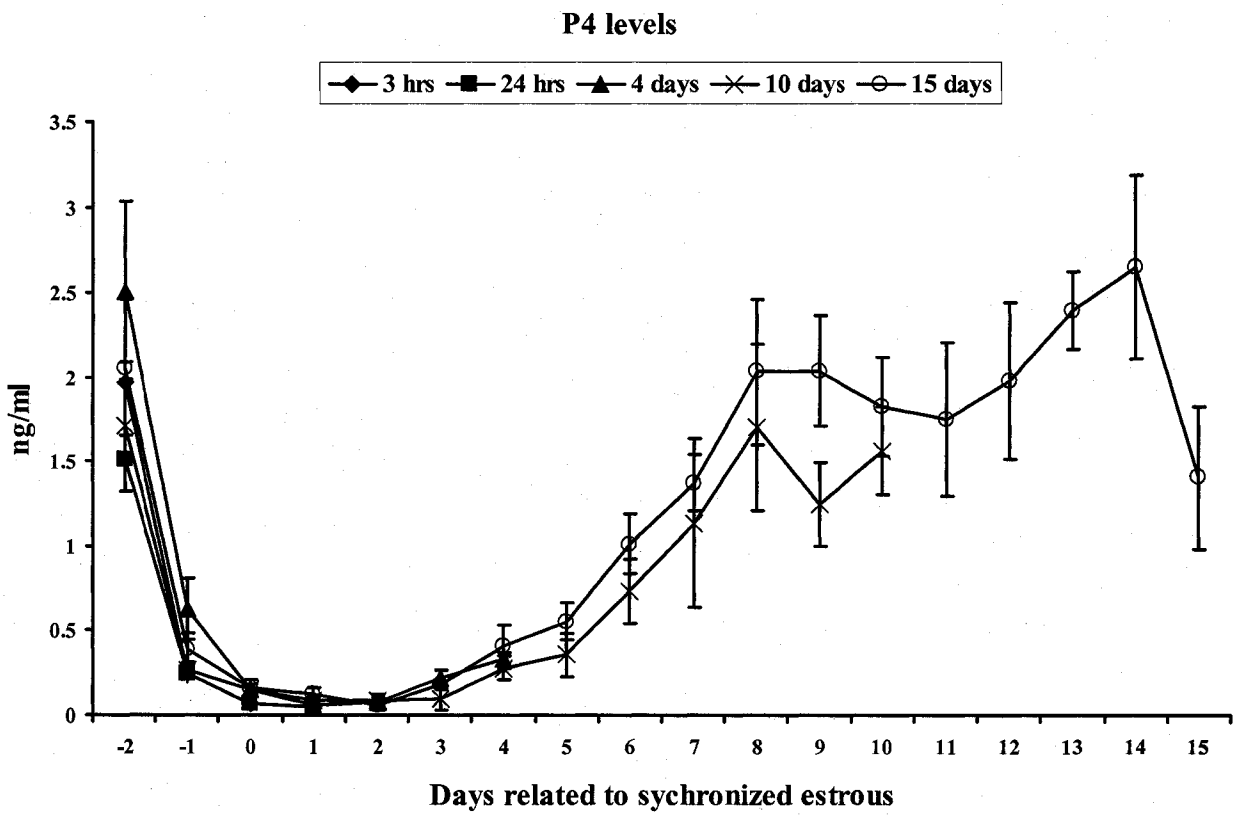


Figure 3.3. Serum concentrations (ng/mL) of P4 in synchronized ewes. Five ewes were assigned to each time period and synchronized with Lutalyse (7.5 mg I.M.) injections two times, 4 h apart. Time periods included 3 and 24 h post estrus and days 4, 10, and 15 of the estrous cycle. Data are means ± S.E. (n = 5 ewes/time period).

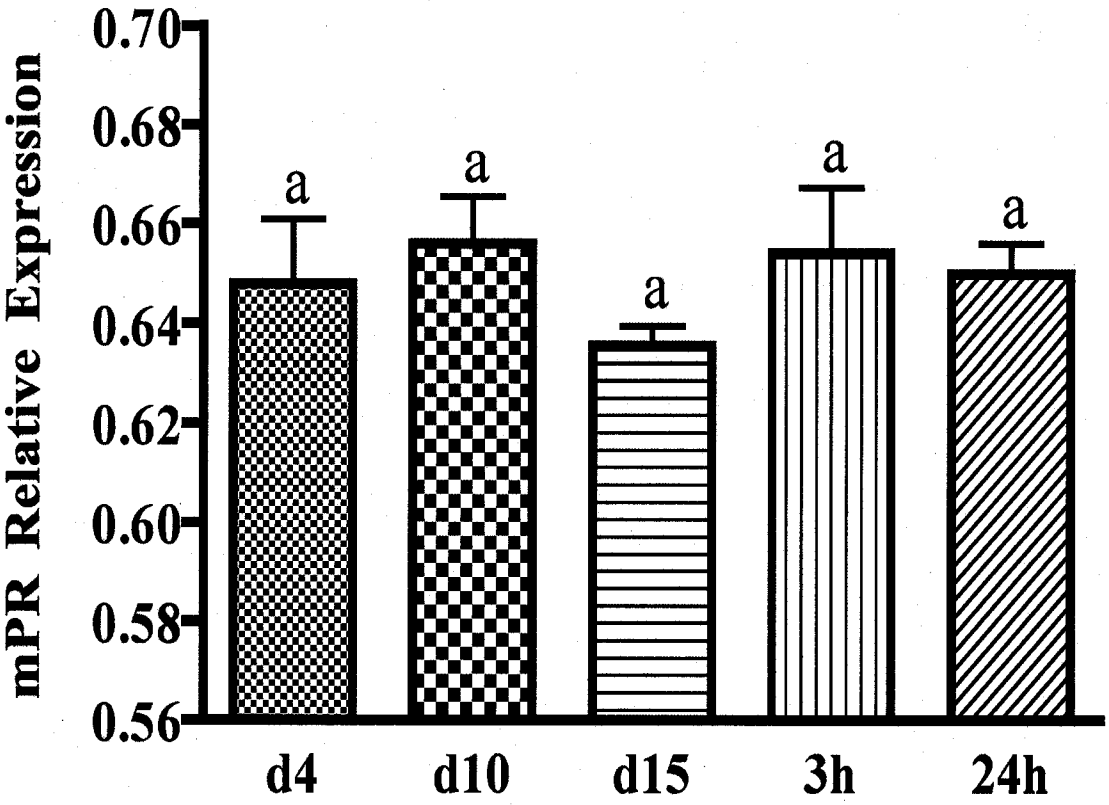


Figure 3.4. Comparison of mean relative expression of mRNA encoding mPR$\alpha$ versus mRNA encoding actin in ovine hypothalamus throughout the estrous cycle (days 4, 10, 15 and 3 and 24 h post estrus). Values are means ± S.E. of a minimum of three sheep.

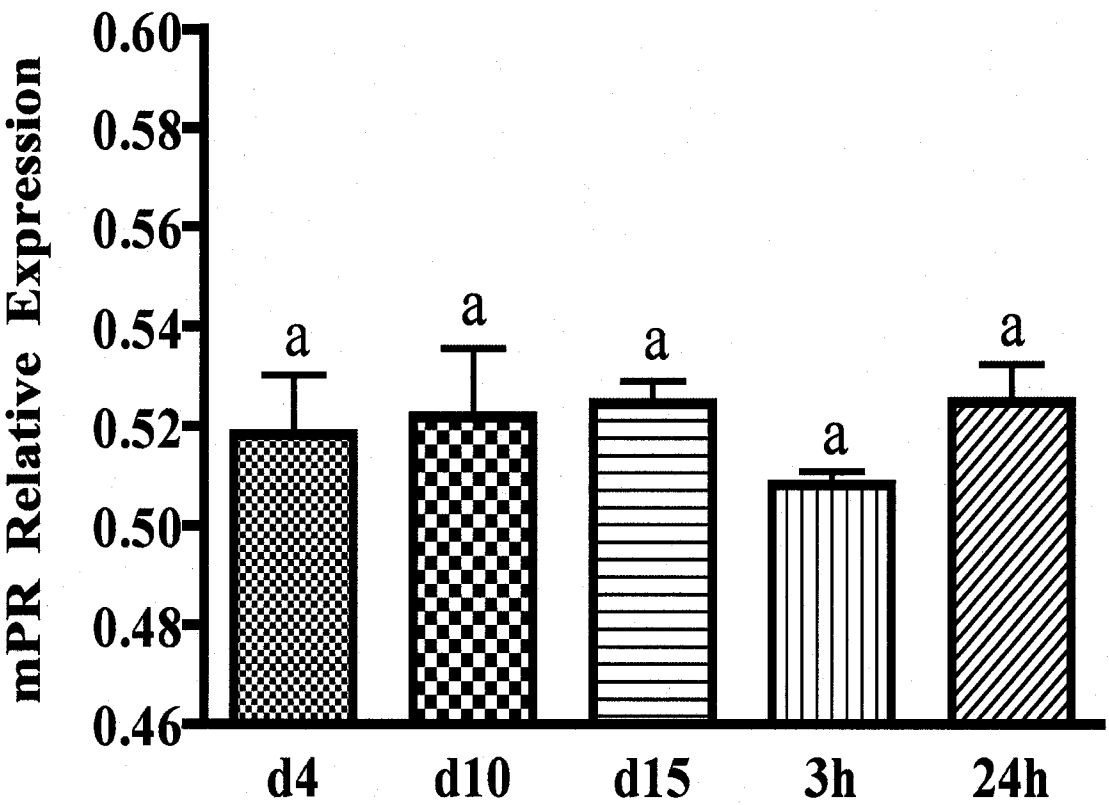


Figure 3.5. Comparison of mean relative expression of mRNA encoding mPR$\alpha$ versus mRNA encoding actin in ovine ovary throughout the estrous cycle (days 4, 10, 15 and 3 and 24 h post estrus). Values are means ± S.E. of a minimum of three sheep.

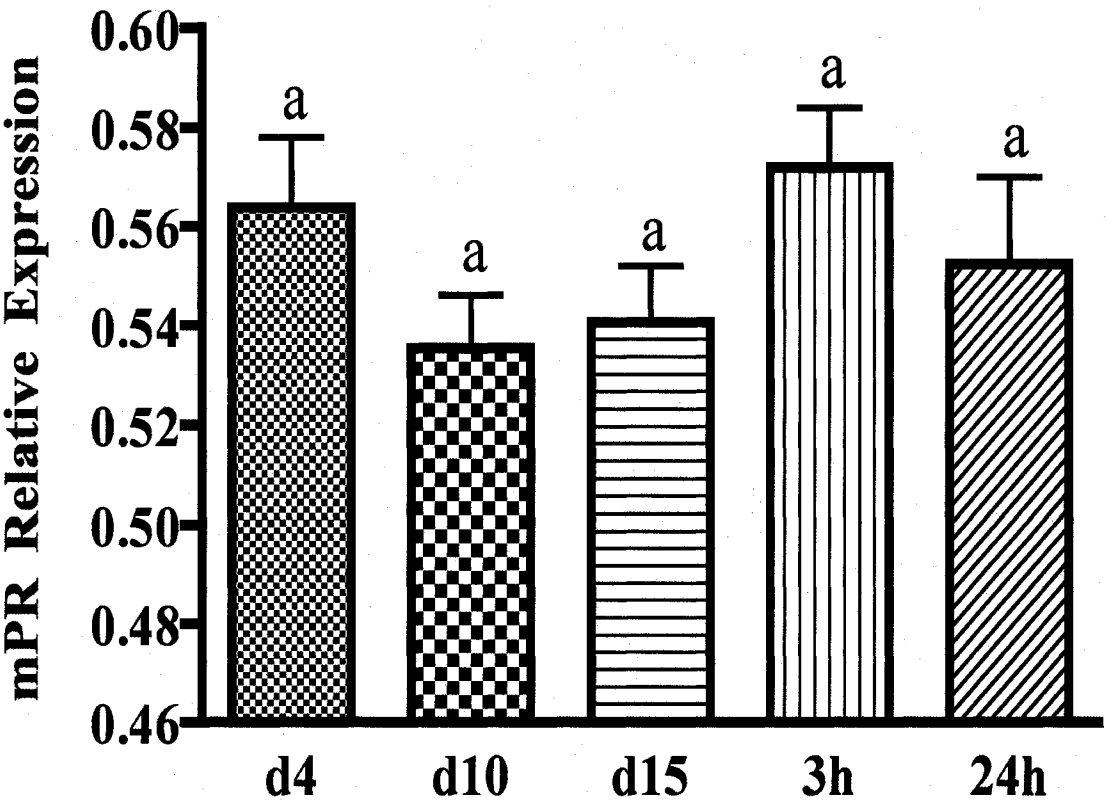


Figure 3.6. Comparison of mean relative expression of mRNA encoding mPR$\alpha$ versus mRNA encoding actin in ovine pituitary throughout the estrous cycle (days 4, 10, 15 and 3 and 24 h post estrus). Values are means ± S.E. of a minimum of three sheep.

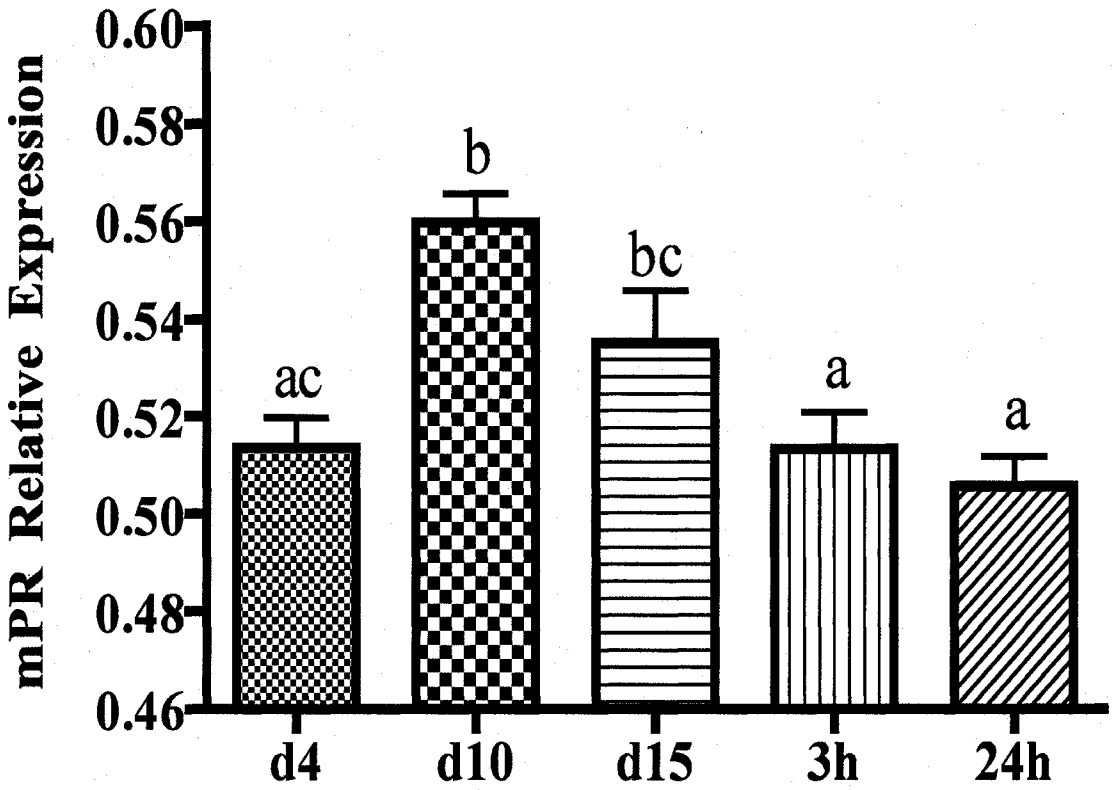


Figure 3.7. Comparison of mean relative expression of mRNA encoding mPR$\alpha$ versus mRNA encoding actin in ovine corpus luteum throughout the estrous cycle (days 4, 10, 15 and 3 and 24 h post estrus). Values are means ± S.E. of a minimum of three sheep. Significant differences ($P < 0.05$) between groups are indicated by different letters above the bars.

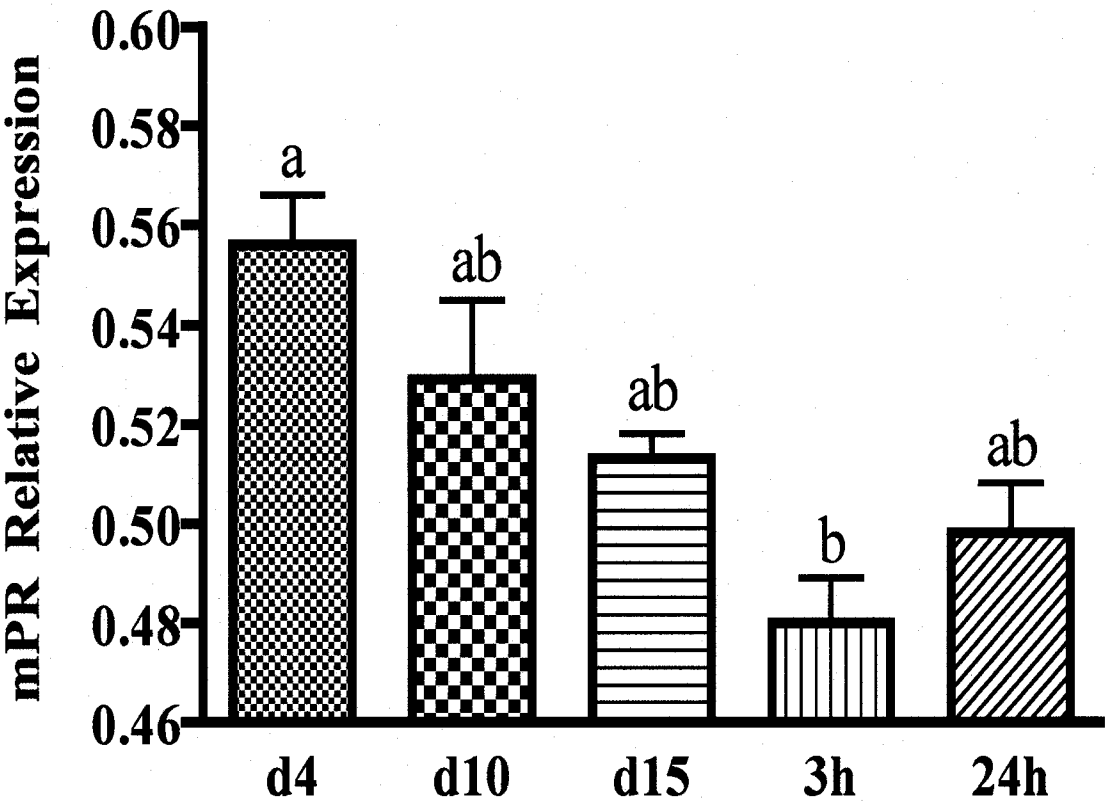


Figure 3.8. Comparison of mean relative expression of mRNA encoding mPR$\alpha$ versus mRNA encoding actin in ovine uterus throughout the estrous cycle (days 4, 10, 15 and 3 and 24 h post estrus). Values are means ± S.E. of a minimum of three sheep. Significant differences ($P < 0.05$) between groups are indicated by different letters above the bars.

Imaging experiments employing confocal fluorescence microscopy revealed that concanavalin A specifically stained the plasma membrane red, yet despite the seven transmembrane domains (based on hydrophobicity analysis) for the putative ovine mPR, no colocalization was observed between mPR-GFP and concanavalin A in CHO cells expressing mPR-GFP (Fig 3.9). Expression of mPR-GFP appeared to be in an intracellular tubular network and possibly in the nuclear envelope. Identical localization of mPR-GFP was observed in both small and large ovine luteal cells (Fig 3.9), as well as pituitary cells (Fig 3.9) all of which express endogenous mPR. Likewise, immunocytochemical localization in CHO cells expressing HA-mPR revealed similar subcellular distribution (Fig 3.10) with no HA-mPR detected in the plasma membrane. Although plasma membrane localization of either mPR fusion protein was not demonstrated using confocal fluorescence microscopy, the putative ovine mPR appeared to reside in a membrane fraction based on analysis of western blots of HA-mPR expressing cells (Fig 3.10). Western blot analysis confirmed that HA-mPR was present in the membrane fraction of HA-mPR transfected Cos7 cells with an estimated molecular weight of 40 kDa. This 40 kDa band was not detected in the membrane fraction from non-transfected cells or in cytosolic fractions of either transfected or non-transfected cells. To ensure bands were specific for the anti-HA antibody, each protein sample was also subjected to the same western blot analysis, but the anti-HA antibody was preabsorbed with HA-peptide. Results from these experiments confirmed the specificity of the bands observed (Fig 3.10).

To determine if mPR-GFP was localized in the endoplasmic reticulum, studies were performed in CHO cells co-transfected with mPR-GFP and pDsRed2-ER, a vector which encodes for a red fluorescent protein that is targeted to the endoplasmic reticulum. Expression of mPR-GFP yielded nearly complete colocalization with the marker for endoplasmic reticulum as depicted in Fig 3.11.

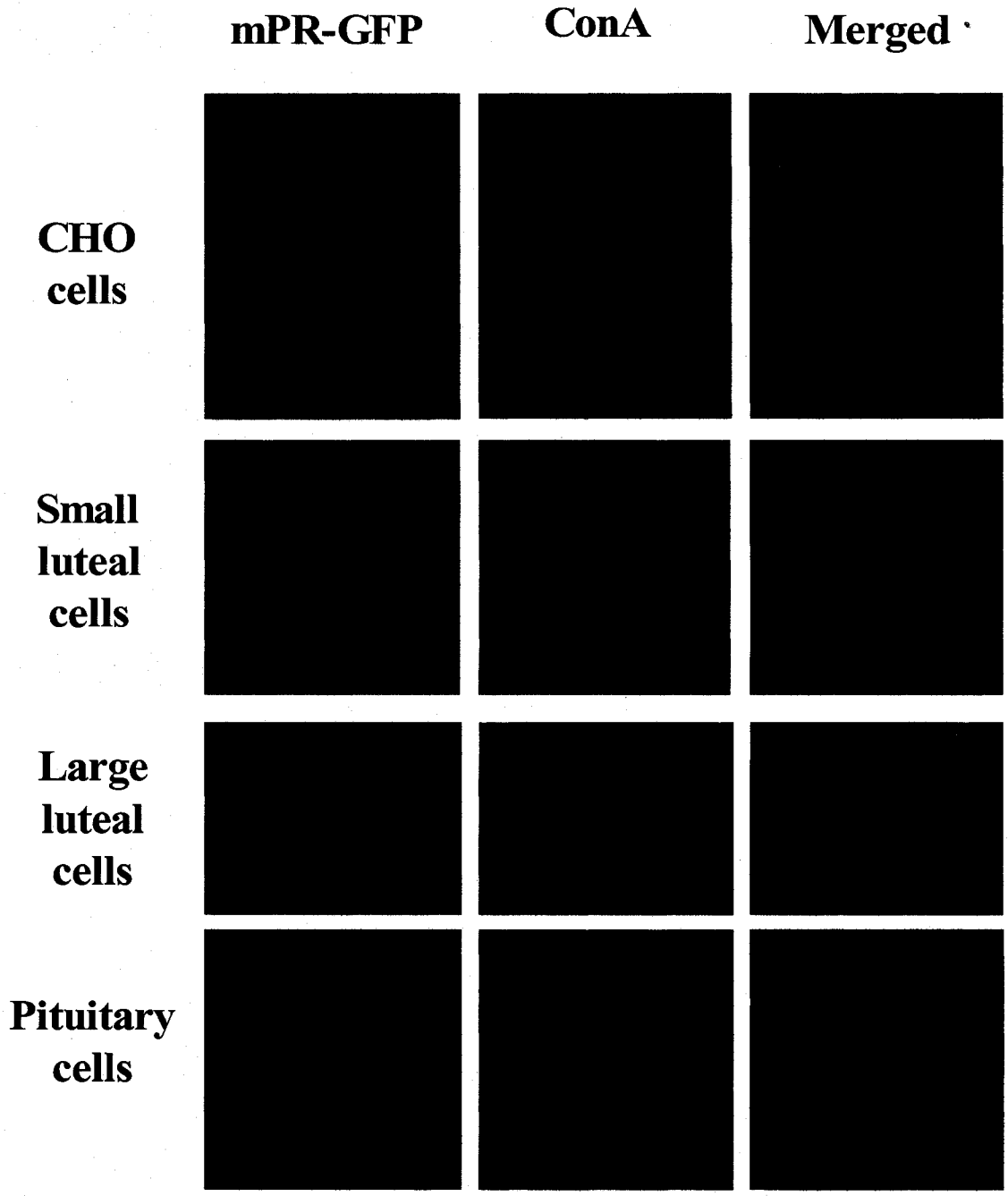


Figure 3.9. Confocal images of mPR-GFP localization relative to plasma membrane. The left panels show confocal images (63 X magnification) of CHO, small luteal, large luteal and pituitary cells expressing mPR-GFP. The plasma membrane is identified by Alexa 594 concanavalin A staining as shown in the middle panel and a merged view of both images is presented in the right panels. Co-localization of the proteins would appear as yellow.

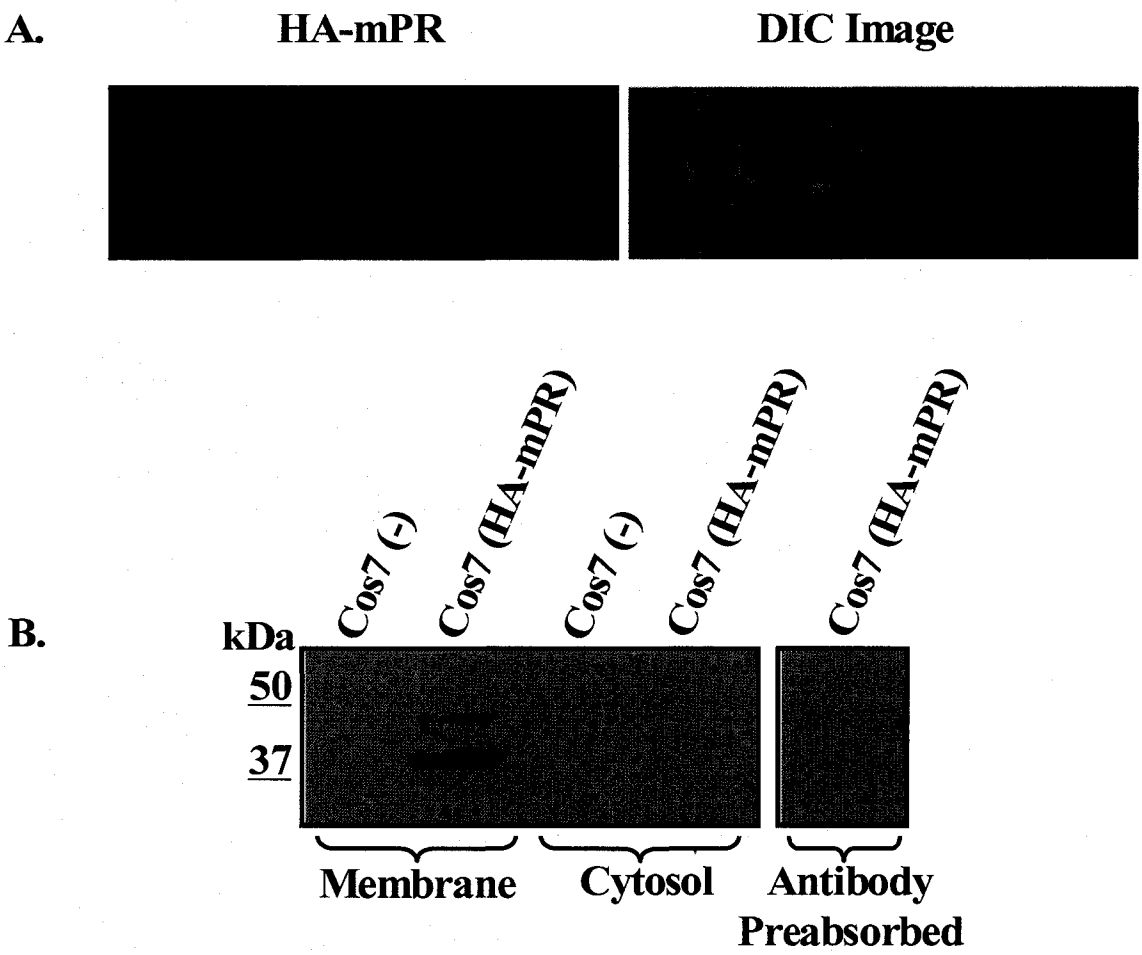


Figure 3.10. A, Confocal images (63 X magnification) of CHO cell expressing HA-mPR, probed with anti-HA antibody and stained with anti rabbit Alexa 488 antibody. Left panel shows HA-mPR localization indicated by green fluorescence with the DIC image in the right panel. Alexa 594 concanavalin A was not used in these experiments as the plasma membrane was disrupted with Triton-X 100. B, Western blot analysis for expression of HA-mPR protein in membrane and cytosolic fractions from Cos7 cells. Non-transfected Cos7 cells and cells expressing HA-mPR were examined. Equal amounts of each fraction were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-HA antibody. To verify specificity of anti-HA antibody, western blots were performed on the membrane fraction of Cos7 cells transfected with HA-mPR, after the anti-HA antibody was preabsorbed with HA-peptide.

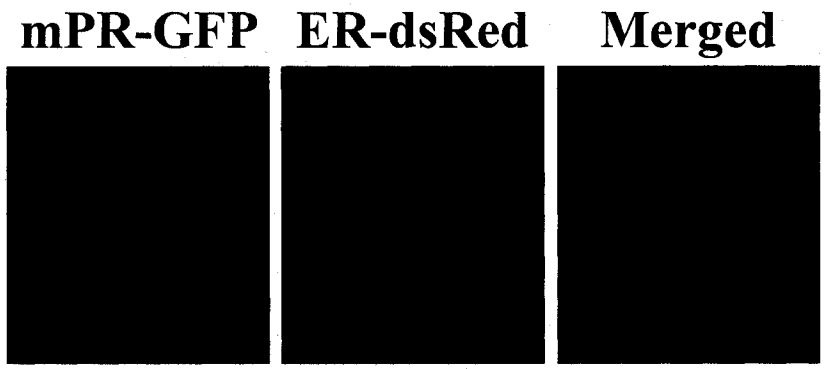


Figure 3.11. Localization of mPR-GFP in the endoplasmic reticulum. Confocal images (63 X magnification) of mPR-GFP coexpressed with pDsRed2-ER, an endoplasmic reticulum targeted red fluorescent protein in CHO cells. Left panel displays expression of mPR-GFP, middle panel expression of pDsRed2-ER, and right panel a merged view of both proteins. Co-localization of proteins is depicted in yellow.

Discussion: As presented in Chapter 2, the isolation and cellular localization of PGRMC1 from ovine CL was described. This protein, despite containing a transmembrane domain, did not display plasma membrane localization based on confocal microscopy. The objective of the current study was to isolate an ovine mPR distinct from the nPR that exhibited plasma membrane localization. Based on data reporting plasma membrane localization of a novel seven transmembrane mPR in seatrout (Zhu, Rice et al. 2003), experiments were conducted to determine if this mPR is expressed in sheep. In this chapter, isolation, identification, tissue distribution, changes in expression throughout the ovine estrous cycle and cellular localization for a unique ovine mPR are discussed. The ovine mPR is distinct from the nPRs in sheep and appears to be a bona fide receptor based on structural characteristics, tissue distribution, steroid binding and activation of an intracellular second messenger. The latter two criteria are presented in chapters 4 and 5, respectively. Pertinent to the current study, the deduced amino acid sequence for the putative ovine mPR protein displays seven transmembrane domains based on hydrophobicity analysis similar to previously identified putative membrane P4 receptors (Zhu, Bond et al. 2003; Zhu, Rice et al. 2003; Zailong and Stocco 2005) supporting the likelihood that this protein is a G-protein-coupled receptor (GPCR). Additionally, the putative ovine mPR has an estimated molecular weight of ~40 kDa, which is within the range expected for GPCRs (Reilander, Reinhart et al. 2000).

Expression of the ovine mPR was localized to reproductive tissues and the hypothalamus and pituitary, which are crucial regulators of reproduction. Nongenomic P4 responses have been reported in each of these tissues (Goodman, Bittman et al. 1981;

O'Byrne, Thalabard et al. 1991). Zhu and coworkers (Zhu, Rice et al. 2003) reported expression of a homologous mPR in the pituitary and ovary of spotted seatrout and these researchers also detected gene expression in the brain and testis. It is quite plausible the ovine mPR is also present in other areas of the brain besides the hypothalamus as well as in the testis; however these tissues were not examined in the present study. The human mPR homologue (mPR$\alpha$) is also expressed mainly in reproductive tissues, particularly in the placenta, testis, ovary, and possibly in the bladder, kidney and adrenal (Zhu, Bond et al. 2003). We, however, did not detect expression of mPR in the kidney and the adrenal was not examined in the present study. In support of expression in the uterus, the recently reported human mPR$\alpha$ homologue was detected in myometrium from pregnant women (Karteris, Zervou et al. 2006). Additionally, expression of mRNA for mPR$\alpha$ was also detected in rat ovary and CL, similar to the ovine mPR$\alpha$ (Zailong and Stocco 2005). These researchers also detected expression in adrenal gland, kidney, brain and lung (Zailong and Stocco 2005). Currently, the differential tissue distribution cannot be reconciled, but may be due to interspecies variation or differences in scientific methodology.

Based on the tissue distribution, with the ovine mPR transcript predominately found in reproductive tissues, another study was completed to investigate the possible changes of mPR expression throughout the ovine estrous cycle in the tissues found to express the mPR. Using real-time quantitative RT-PCR, no significant changes were observed across the estrous cycle in the hypothalamus, ovary or pituitary. With regard to the CL, mRNA for the mPR was significantly ($P < 0.01$) higher in CL from sheep on d

10 compared to d 4 of the cycle as well as 3 h and 24 h post estrus. The next highest expression of mPR in the CL was on d 15 which was significantly ($P < 0.05$) higher compared to 3 h and 24 h post estrus. Interestingly, the expression of mPR appears to be regulated in a manner that coincides with the capacity of the CL to synthesize P4. Comparing serum concentrations of P4 (Fig 3.3) to the expression of mPR in the CL (Fig 3.7) it is evident that expression of mPR closely mimics serum P4 concentrations suggesting that the ovine mPR is possibly regulated by P4 and that the mPR plays a central role in the regulation of CL function. Further, it is important to note, that CL samples taken from sheep at 3 and 24 h post estrus in actuality represent the corpus albicans. Relative expression of the mPR appeared lower in the corpus albicans samples providing further support for a functional role for mPR in the CL. The only other data available which investigated mPR expression in the CL is from the rat (Cai and Stocco 2005). These researchers however, determined the mRNA changes throughout pregnancy in the rat CL and observed that mPR$\alpha$ expression increased with advancing gestation and then dramatically decreased prior to parturition. In support of our data, the expression of mPR$\alpha$ in the rat CL also mimicked serum concentrations of P4 (Cai and Stocco 2005) further suggestive that mPR plays a key role in CL function.

As with the changes in levels of expression of mPR in the CL, very little data exist pertaining to changes in expression of the mammalian mPR$\alpha$ in other tissues. Karteris et al. (Karteris, Zervou et al. 2006) recently demonstrated that P4 and estradiol (100 nM) significantly induced expression of mRNA for human mPR$\alpha$ in human myometrial cells. In the current study, we observed the highest levels of expression in

uterine mRNA for ovine mPR on d 4 of the estrous cycle which was significantly ($P <$ 0.01) higher compared to 3 h post estrus with an approximate mRNA increase of 4 fold. Although not significantly different, the expression of mPR appears to decrease with advancement throughout the estrous cycle (Fig 3.8). The difference in expression of mPR noted between d 4 and 3 h post estrus may be due to the ratio of circulating P4 and estradiol since at 3 h post estrus more estradiol would be present in circulation than P4 compared to d 4 of the cycle when estradiol is low and serum concentrations of P4 would be increasing. Whether the difference in expression of the ovine mPR throughout the estrous cycle is due to the serum concentrations of P4 and estradiol is not known.

Despite the presence of seven transmembrane domains, the ovine mPR does not appear to localize to the plasma membrane based on confocal microscopy of two different mPR fusion proteins. Our initial studies employing the mPR-GFP construct displayed fluorescence distinctly around the nucleus, possibly in an intracellular tubular network. These early studies were surprising as localization of the ovine mPR in the plasma membrane was expected, due to the seven transmembrane domains and the plasma membrane localization of the seatrout mPR in oocytes (Zhu, Rice et al. 2003). Experiments were also conducted with other cell lines expressing the mPR-GFP fusion protein to confirm that the localization of mPR-GFP was not due to cell lineage. In each case subcellular localization appeared to be within an intracellular membrane. As the ovine mPR is expressed in the pituitary and CL, the cellular localization of mPR-GFP in transfected pituitary and luteal cells was also examined. Once again, there was clear perinuclear expression of mPR-GFP. As GFP is a large protein, it is possible that the

tertiary structure of the ovine mPR was altered, which interfered with the final cellular destination in transfected cells. To address this issue the HA-mPR construct was generated, which consists of three hemaglutinin (HA) peptides (27 amino acids) on the N-terminus of the ovine mPR. With the three HA peptides, the HA-mPR construct contains a much smaller tag compared to GFP plus the tag is on the other end of the receptor compared to the mPR-GFP construct which has the GFP on the C-terminus. Despite the differences between the two different fusion proteins, cellular localization was almost identical between the two as immunocytochemical localization of HA-mPR was similar to the mPR-GFP fusion protein. Thus, colocalization with a plasma membrane marker (concanavalin A) was not evident for either HA-mPR or mPR-GFP. I should note that while our data do not suggest plasma membrane localization, this interpretation differs from that of Karteris et al., (Karteris, Zervou et al. 2006). These researchers concluded that the human mPR homologue is localized to the plasma membrane. Clearly, our studies utilized a receptor from a diiferent species. Thus, there are possible species differences. It is important to underscore that my data do not eliminate the possibility of plasma membrane expression but certainly suggest prominent localization to an intracellular compartment, likely the endoplasmic reticulum. Interestingly, a similar pattern of intracellular distribution is evident in the data presented by Karteris et al., (Karteris, Zervou et al. 2006). In fact, the authors stated that the mPR localized with nuclear receptor in the cytoplasm. Thus, as in the case of plasma membrane expression, the data presented by Karteris et al., (Karteris, Zervou et al. 2006)

cannot be used to exclude an intracellular site of action such as endoplasmic reticulum membrane.

Exploiting the HA epitope, western blots were performed on membrane and cytosolic preparations of cells expressing HA-mPR and non-transfected cells to further elucidate localization of the ovine mPR. A band of approximately 40 kDa, the estimated molecular weight of the ovine mPR, was detected only in the membrane fraction from cells transfected with HA-mPR. It is noteworthy that the membrane preparation used in these experiments contains all cellular membranes, not just the plasma membrane. Additionally, a higher molecular weight band was also detected in the membrane fraction from cells transfected with HA-mPR and appeared to be specific for the anti-HA antibody as detection of both bands was absent when the antibody was preabsorbed with HA peptide (Fig 3.10). It is possible that the higher molecular weight band represents a glycosylated form of the ovine mPR as there are two possible O-linked glycosylation sites at amino acid positions 29 and 34.

Results from confocal microscopy utilizing CHO cells expressing both mPR-GFP and ER-dsRed provided evidence that the ovine mPR is an intracellular transmembrane receptor. Nearly complete colocalization of mPR-GFP with the endoplasmic reticulum marker was observed in these studies. In support of the cellular localization of ovine mPR, Revankar et al., (Revankar, Cimino et al. 2005) recently reported that GPR-30, a GPCR that binds estradiol is located primarily in the endoplasmic reticulum and elicits $Ca^{2+}$ mobilization upon estradiol treatment. A functional intracellular receptor requires ligand passage across the plasma membrane. As P4 can easily cross the plasma

membrane, it seems quite conceivable a functional ovine mPR may be located in the endoplasmic reticulum.

In summary, chapters 2 and 3 describe the isolation of two different proteins (PGRMC1 and mPR$\alpha$) expressed in the sheep which display localization predominately in the endoplasmic reticulum. The cellular localization of PGRMC1 in the endoplasmic reticulum was not surprising as other laboratories have demonstrated similar results (Falkenstein, Schmieding et al. 1998). However, localization of the ovine mPR in the endoplasmic reticulum was not expected as this protein was reported to be a plasma membrane receptor (Zhu, Rice et al. 2003). Despite the working hypothesis that the nongenomic effects of P4 were initiated through a plasma membrane receptor, results from the current study demonstrating a possible GPCR localized to the endoplasmic reticulum provides a novel mode of action in which P4 may function. As such, my working hypothesis was modified and the remaining studies presented in this dissertation were aimed at deciphering the steroid binding characteristics and possible functions of the ovine mPR.

## CHAPTER IV.

## STEROID BINDING CHARACTERISTICS OF THE OVINE mPR

Introduction: Despite the homology to other reported mPRs, information pertaining to the steroid binding characteristics of the ovine mPR was lacking. An essential criterion to verify the putative ovine mPR as a true receptor was to identify the ligand(s) that bind to the ovine mPR and to determine the binding kinetics of this putative receptor. The first mPR isolated was from seatrout and these researchers utilized soluble recombinant protein produced in BL21 *E. coli* cells to determine the steroid binding characteristics (Zhu, Rice et al. 2003). Saturable, specific binding of P4 was obtained with the recombinant protein and the presence of a single binding site ($K_d$ 30 nM) was demonstrated with Scatchard analysis (Zhu, Rice et al. 2003). Competition studies with a variety of steroids revealed binding was highly specific for P4 and 17$\alpha$-hydroxyprogesterone. Similar results were obtained with recombinant human mPR$\alpha$, mouse mPR$\beta$ and human mPR$\gamma$ produced in an *E. coli* expression system (Zhu, Bond et al. 2003).

Use of recombinant protein for binding studies provides researchers with a fairly straightforward method to evaluate interaction between ligand and receptor. Either the recombinant protein binds the ligand of interest or it does not. However, limitations exist with this system as the protein synthesized may not posses the tertiary structure it would normally have *in vivo* and may lack any post-translational modifications that would be present normally. As such, the binding characteristics of the reported mPRs may be

different *in vivo* than those obtained using a soluble form of the receptor. A paper investigating the regulation of progestin membrane receptors in the rat CL was recently published which provides a better understanding of the binding characteristics of the mammalian mPRs (Cai and Stocco 2005). The rat serves as a good model for these studies because nuclear PRs have not been identified in rat CL. [$^3$H]-progesterone binding was determined in subcellular fractions of corpora lutea obtained from rats on d-14 of pregnancy. These researchers demonstrated that P4 and 20$\alpha$-hydroxyprogesterone competed for binding whereas a much higher concentration of 17$\alpha$-hydroxyprogesterone was required to inhibit binding of the radioligand (Cai and Stocco 2005). Further, a $K_d$ value of 162 nM was reported. These binding studies were performed with luteal membranes; thus the results may provide a better representation of the binding kinetics *in vivo*. These researchers also reported the presence of five genes expressed in the rat CL previously postulated to encode for putative membrane receptors for P4 (Cai and Stocco 2005). Thus, it is difficult to verify which protein correlates with the binding data.

Consequently, to elucidate the steroid binding characteristics of the ovine mPR, binding studies were performed using crude membrane fractions from CHO cells expressing the sheep mPR. CHO cells were selected because they do not express mPR$\alpha$ (as determined by the lack of mRNA for mPR) or the nuclear P4 receptors. Experiments were conducted to test the hypothesis that *P4 is the true ligand for the ovine mPR.*

Materials and Methods

Receptor Binding Assays. A crude membrane fraction was prepared from CHO cells and CHO cells transfected with cDNA for ovine mPR following procedures as described (Soloff and Sweet 1982) with slight modifications. CHO cells were cultured in DMEM, supplemented with 10% FBS, non-essential amino acids (8.9 mg/L L-Alanine, 15 mg/L L-Asparagine, 13.3 mg/L L-Aspartic acid, 14.7 mg/L L-Glutamic acid, 7.5 mg/L Glycine, 11.5 mg/L L-Proline and 10.5 mg/L L-Serine) and penicillin-streptomycin (100 I.U. penicillin and 100 μg/mL streptomycin) and transfected with a vector (pcDNA3.1+) encoding ovine mPR using the Polyfect procedure (QIAGEN, Valencia, CA). At 48h post-transfection, transfected and non-transfected CHO cells were washed with cold PBS and removed from tissue culture plates using a cell scraper and cold PBS, and concentrated by centrifugation. Cells were washed again with cold PBS, counted using a hemacytometer and homogenized in homogenization buffer (100 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-HCL, 1mM EGTA, plus protease inhibitors; pH 7.2) with a QIAshredder (QIAGEN, Valencia, CA) per manufacture's instructions. Supernatants were collected and spun at 100,000 x g at 4°C for 1h. Supernatants were removed and pellets containing the membrane fractions were resuspended in cold sample buffer (10 mM Tris-HCL, 250 mM sucrose, 1mM EGTA, plus protease inhibitors; pH 7.2). An aliquot of the membrane fractions from transfected and nontransfected cells was kept separate and protein concentration was determined using the Coomassie Plus Protein Assay Kit (Pierce; Rockford, IL). Duplicate aliquots of membrane fractions from CHO cells transfected with mPR and from nontransfected CHO cells were incubated at 4°C for 1 h in 0.3-mL

TEDG buffer (10mM Tris-HCl, 1.5mM EDTA, 1mM Dithiothieitol, 10% Glycerol; pH 7.6) containing 4 nM $^{3}$H-progesterone ($^{3}$H-P4) and digitonin (250 μM). The bound and free ligands were separated by the addition of 0.8 mL ice cold dextran-coated charcoal (0.3 g defined charcoal and 0.03 g Dextran [Sigma, St Louis, MO] in 100 mL of TEDG buffer) and incubation on ice for 10 min. After centrifugation at 1100 x g for 15 min at 4°C, 0.9 mL supernatants were carefully removed, mixed with 5 mL of ScintiSafe scintillation cocktail (Fisher Chemical, Fair Lawn, NJ) and radioactivity was quantified in a Beckman scintillation spectrometer. Non-specific binding was measured in duplicate in the presence of 4 μM nonradioactive progesterone. Other binding experiments were performed with increasing concentration of protein and increasing incubation time. Additional controls included tubes without cell membrane fractions but with $^{3}$H-P4 and digitonin. To test the specificity of binding for the ovine mPR, binding of $^{3}$H-P4 was measured in the absence or presence of increasing concentrations of several steroids. Statistical analysis of the results from the competition binding studies were performed using the Newman-Keuls Multiple Comparison Test in Prism (Version 4a, from GraphPad Software, Inc.) and significance was taken as a value of $P < 0.05$. When variances were not homogenous, data were transformed by Log 10 function.

Saturation binding experiments and competition curves: A stable cell line which expresses the ovine mPR was generated for the binding studies involving ligand saturation and competition curves. CHO cells were transfected with a vector (pcDNA3.1+) encoding ovine mPR using the Polyfect procedure (QIAGEN, Valencia, CA) according to manufacturer's instructions. Twenty four hours after transfection, the

medium was changed, and G418 (Mediatech, Inc., Herndon, VA) was added to a final concentration of 400 μg/mL. After 2 weeks of selection in G418, the cells were trypsinized and serial diluted in complete medium plus G418, plated into wells of a 96-well tissue culture plate, and incubated until single colonies became visible. Each well that contained a single colony was grown to confluence at which time colonies were trypsinized and transferred to a single well of a 24-well tissue culture plate. Once these colonies reached confluence, individual colonies were trypsinized and each colony was cultured in a single well of a 6-well tissue culture plate. The same procedure was performed again, but colonies were seeded onto a 90 mm tissue culture dish. Expression of the ovine mPR was determined in each colony by RT-PCR using procedures described in Chapter 3. CHO cells not expressing ovine mPR served as a negative control for the RT-PCR. Membrane fractions from the stable CHO cell line expressing ovine mPR were incubated with increasing concentrations of $^{3}$H-P4 for the saturation binding experiments. The binding studies were performed as described above, except that the incubation time was increased to 4 h at 4°C based on the time course experiments. Data from the saturation experiments were analyzed by nonlinear regression analysis using GraphPad Prism (Version 4a, from GraphPad Software, Inc.). Binding of $^{3}$H-P4 was also measured in the absence or presence of increasing concentrations of P4, 20α-hydroxyprogesterone or 17α-hydroxyprogesterone to determine the half-maximal inhibitory concentration ($IC_{50}$) of these progestins. The $IC_{50}$ of the progestins was obtained by nonlinear fitting of inhibition curves using GraphPad Prism (Version 4a, from GraphPad Software, Inc.).

Western Blot Analysis. The stable cell line decribed above (mPR-CHO) was utilized for the western blot analysis. Crude membrane preparations from the mPR-CHO cells were generated as described for the receptor binding assays. Additionally, a plasma membrane fraction was also isolated from mPR-CHO cells using similar methods described for the crude membrane preparation with slight modifications. To obtain the plasma membrane fraction, after the pellet containing the crude membrane fraction was collected via centrifugation, the pellet was subsequently resuspended in 1 mL of 10% sucrose (made in homogenization buffer). This fraction was then layered over a 28% sucrose mixture in ultra clear tubes and centrifuged at 40,000 x g at 4°C for 0.5h. The membrane fraction was removed at the interface of the 10% and 28% sucrose layers and centrifuged at 100,000 x g at 4°C for 0.5h. The pellet containing the plasma membrane was resuspended in sample buffer and stored at -80°C until western blot analysis.

For analysis of transferrin receptor and calreticulin, equal amounts of lysate from the crude membrane and plasma membrane fraction were separated using denaturing PAGE followed by transfer to nitrocellulose membranes for electroblotting. Membranes were blocked in TBS-T (140 mM NaCl, 10 mM Tris, 0.1% Tween 20, pH 7.4) + 5% non-fat milk for 1h at room temperature. The membranes were incubated overnight at 4°C on an orbital shaker with an antibody (1:1000 dilution) specific for transferrin receptor obtained from Zymed Laboratories (San Francisco, CA) or with an antibody (1:1000 dilution) specific for calreticulin purchased from Affinity Bioreagents (Golden, CO). Blots were then washed for 20-30 min with TBS-T before incubating for 1 h in 1% milk made in TBS-T with anti-mouse IgG-HRP (1:2000 dilution) antibody for the transferrin

receptor or an anti-rabbit IgG-HRP (1:2000 dilution) antibody for the calreticulin. Again membranes were washed for 20-30 min and proteins were visualized by chemiluminescence (SuperSignal West Femto Maximum Sensitivity Substrate; Pierce, Rockford, IL).

Results: As shown in Fig 4.1, binding of $^{3}$H-P4 increased with increasing amounts of membrane fractions from mPR-CHO cells. In contrast little binding was observed in the membrane fractions from CHO cells. Likewise, binding of $^{3}$H-P4 increased with incubation time reaching a plateau at approximately 4 h at 4°C (Fig 4.2). A variety of steroids were tested (1000-fold excess) for their abilities to displace binding of $^{3}$H-P4 to crude membrane fractions of mPR transfected CHO cells (Fig 4.3). Only excess P4, 20$\alpha$-hydroxyprogesterone and 17$\alpha$-hydroxyprogesterone were able to significantly ($P <$ 0.001) displace binding of radiolabeled P4. Estradiol, testosterone, cortisol and the P4 antagonist RU486 failed to inhibit binding of $^{3}$H-P4 to crude membranes from CHO cells expressing mPR (Fig 4.3). In Fig 4.4, a representative saturation curve of $^{3}$H-P4 binding to membrane fractions from mPR-CHO cells is shown. The average $B_{max}$ and $K_d$ values of three separate experiments were 624 ± 119 fmol/μg protein and 122 ± 50 nM, respectively.

Based on the competition binding studies additional experiments were performed to more clearly evaluate the abilities of P4, 20$\alpha$-hydroxyprogesterone and 17$\alpha$-hydroxyprogesterone to displace binding of radiolabeled P4 to crude membranes from mPR-CHO cells (Fig 4.5). Low levels of P4 ($IC_{50}$ = 174 nM) competed for binding of

the tracer in a dose-dependent manner (Fig 4.5), whereas higher concentrations of 20α-hydroxyprogesterone ($IC_{50}$ = 298 nM) or 17α-hydroxyprogesterone ($IC_{50}$ = 735 nM) were required to reduce binding by 50% (Fig 4.5).

An additional experiment was conducted, in which the plasma membrane fraction was isolated from CHO cells stably expressing the mPR and subsequently used in the receptor binding assays. Despite the predominant localization of the ovine mPR in the endoplasmic reticulum as discussed in Chapter 3, there was still displaceable binding of radiolabeled P4 by unlabeled P4 in the plasma membrane fraction (data not shown). To determine if the plasma membrane fraction was actually purified plasma membrane or if it contained intracellular membrane contamination, western blot analysis was employed. Specifically, both the crude membrane and plasma membrane fractions isolated from mPR-CHO cells were subjected to SDS-PAGE and then probed with an antibody specific to a plasma membrane specific protein or an endoplasmic reticulum specific protein, transferrin receptor or calreticulin, respectively. As expected, both the transferrin receptor and calreticulin were detected in the crude membrane fraction as shown in Figure 4.6. Likewise, the transferrin receptor was detected in the plasma membrane fraction. However, calreticulin was also present in the plasma membrane fraction (Fig 4.6). From these data it appears the plasma membrane fraction utilized in our receptor binding assays contains some intracellular membranes, specifically the endoplasmic reticulum, which probably accounts for the displaceable binding of P4 observed.

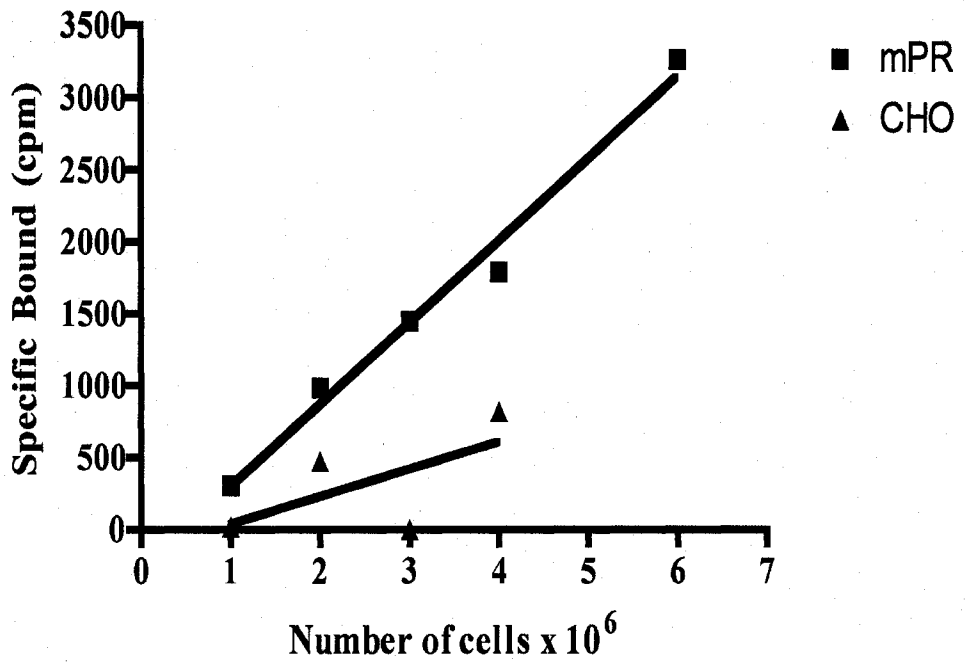


Figure 4.1. Binding of $^{3}$H-progesterone was measured with increasing amounts of crude membranes from CHO cells and CHO cells expressing mPR. Displacement of $^{3}$H-progesterone was measured in the presence of 1000-fold excess nonradioactive progesterone. Values are a representation of three individual experiments.

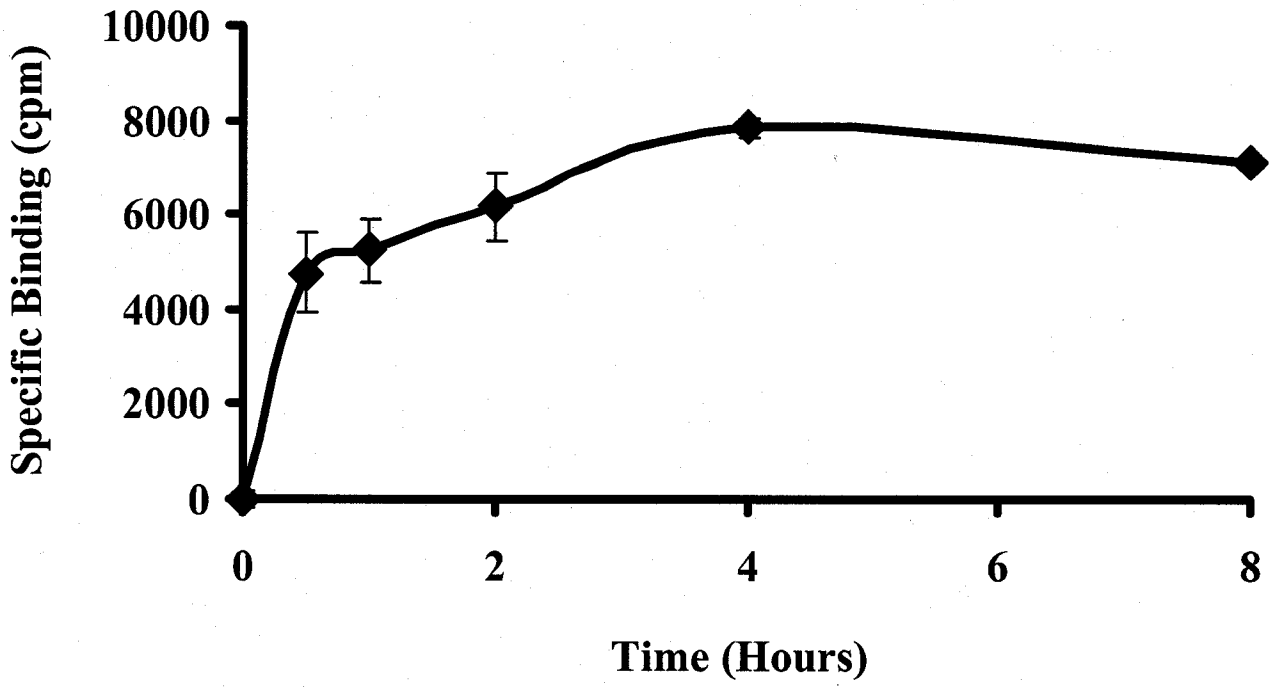


Figure 4.2. Binding of $^{3}$H-Progesterone in crude membrane fractions from CHO cells expressing the ovine mPR at various times of incubation. Displacement of $^{3}$H-progesterone was measured in the presence of 1000-fold excess nonradioactive progesterone. Values are means ± S.E. (n = 4).

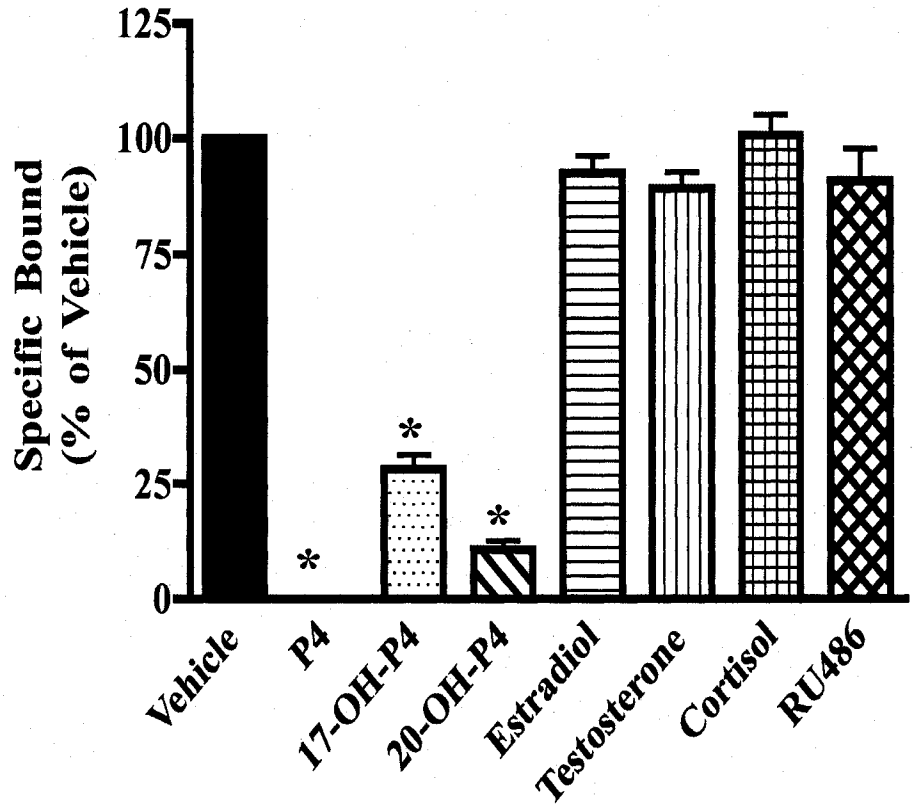


Figure 4.3. Displacement of $^{3}$H-progesterone from crude membranes of CHO cells expressing mPR in the presence of vehicle or 1000-fold excess nonradioactive steroids. Steroids included progesterone (P4), 17$\alpha$-hydroxyprogesterone (17-OH-P4), 20$\alpha$-hydroxyprogesterone (20-OH-P4), estradiol, testosterone, cortisol, or RU486. Values are means ± S.E. (n = 3). *$P < 0.001$, compared to vehicle control.

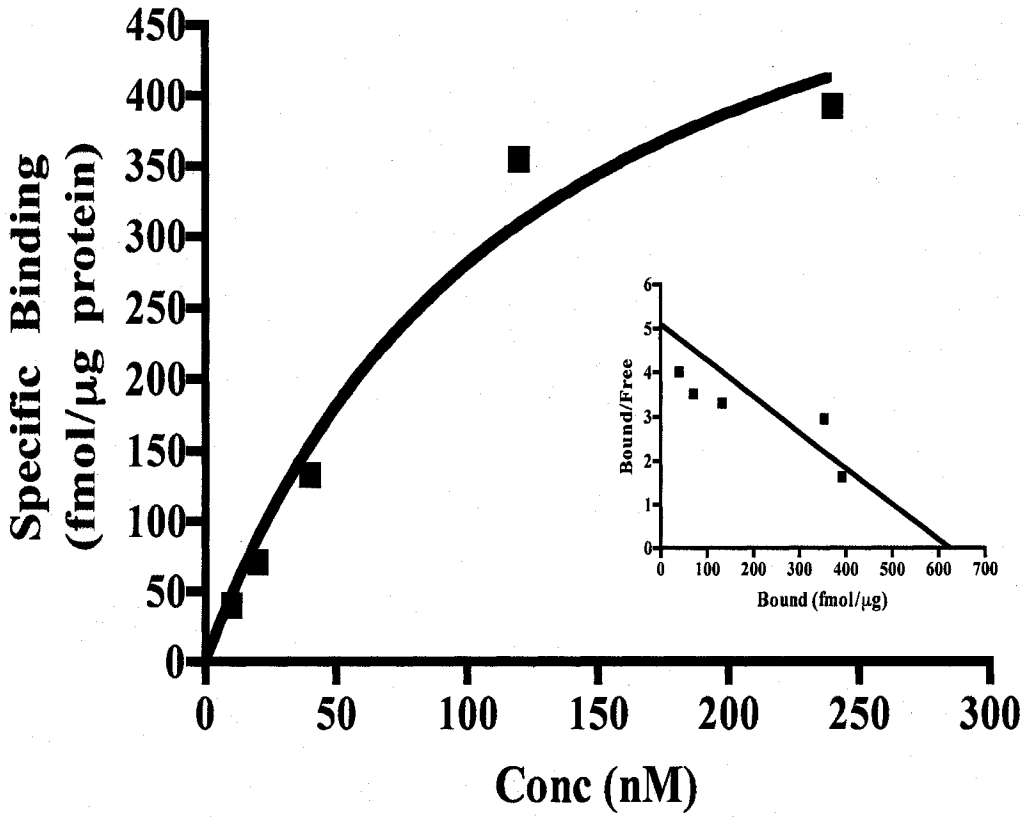


Figure 4.4. Representative saturation curve in the presence of increasing concentrations of $^{3}$H-progesterone. The $B_{max}$ and $K_d$ values of $^{3}$H-progesterone binding were 624 fmol/µg protein and 122 nM, respectively. The *inset* shows Scatchard analysis of the same data.

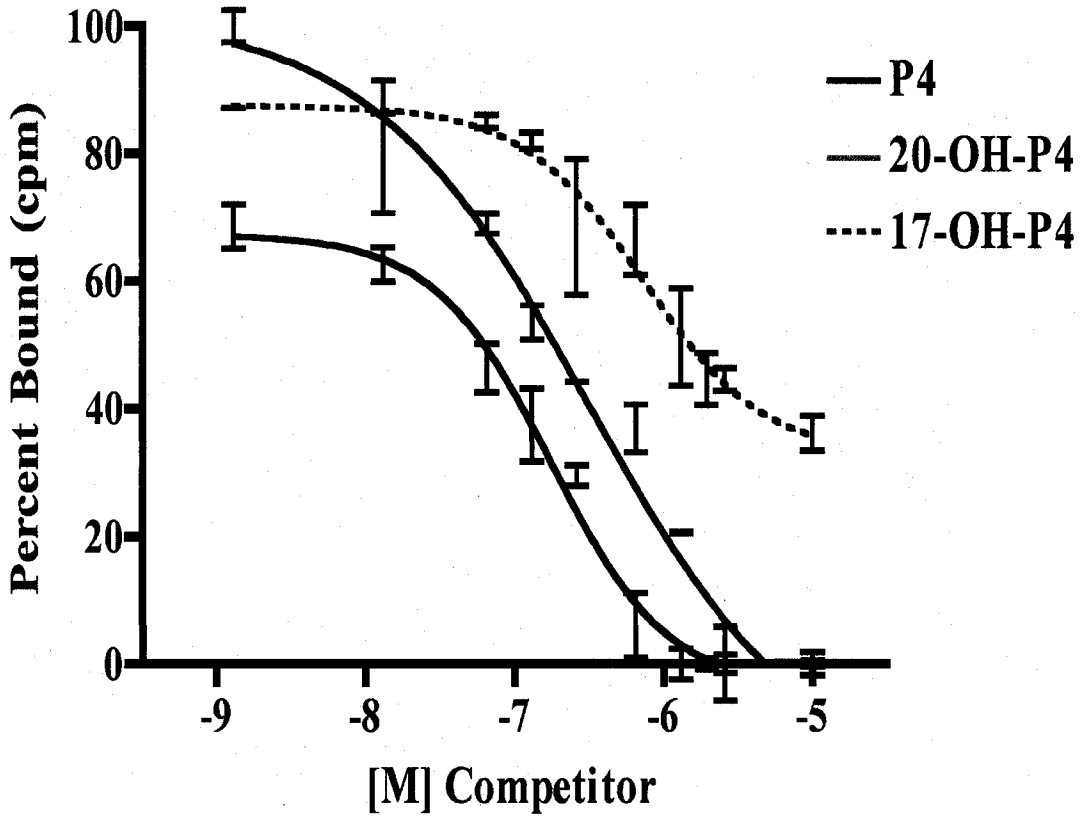


Figure 4.5. Specificity of progestin binding sites for ovine mPR. Binding of $^{3}$H-progesterone was measured in the absence or in the presence of increasing concentrations of nonradioactive progestins. Progestins evaluated included progesterone (P4), 20$\alpha$-hydroxyprogesterone (20-OH-P4) and 17$\alpha$-hydroxyprogesterone (17-OH-P4). Each point represents the average ± S.D. of progestin binding expressed as a percentage of maximum $^{3}$H-progesterone binding.

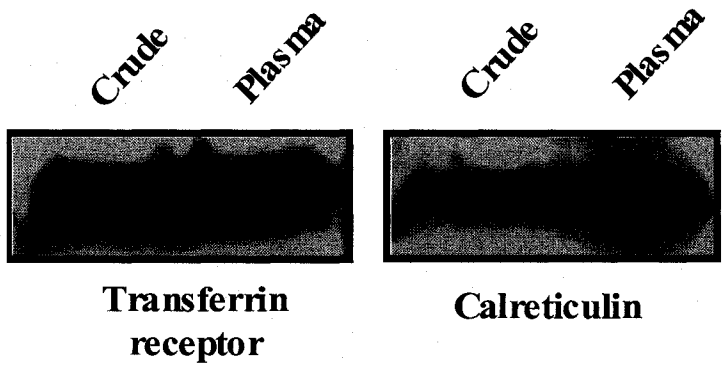


Figure 4.6. Western blot analysis for expression of transferrin receptor or calreticulin protein in crude membrane and plasma membrane fractions from mPR-CHO cells. Equal amounts of each fraction were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-transferrin receptor antibody or anti-calreticulin antibody.

Discussion: The studies described in this chapter demonstrate that the ovine mPR specifically binds only progestins. Progesterone, 20$\alpha$-hydroxyprogesterone and 17$\alpha$-hydroxyprogesterone significantly ($P < 0.001$) displaced binding of $^{3}$H-P4 to membrane fractions from CHO cells expressing ovine mPR. Similar to the results presented in this chapter, the seatrout mPR$\alpha$ also exhibits specific binding for P4, 20$\beta$-hydroxyprogesterone and 17$\alpha$-hydroxyprogesterone (Zhu, Rice et al. 2003). The other steroids tested did not compete for $^{3}$H-P4 binding further supporting progestins as the true ligand for the ovine mPR. It is important to note the membrane fractions used in the present study contain membranes from the plasma membrane as well as other cellular organelles. Specific binding sites for P4 have been detected in microsomal rich fractions in a variety of mammalian species. Bramley and Menzies (Bramley and Menzies 1994) reported P4 binding sites in ovine CL that are unlike classical nuclear PRs in that they are enriched in intracellular membrane fractions and not found associated with the cytosolic or nuclear fractions. RU486, the nuclear PR antagonist did not block P4 binding to intracellular membranes suggestive of a PR unlike the nPR (Bramley and Menzies 1994). In the present study RU486 also failed to compete for $^{3}$H-P4 binding in membrane fractions from CHO cells expressing ovine mPR. Similar results have been reported in microsomal fractions of corpora lutea from cows (Rae, Menzies et al. 1998), pigs (Bramley and Menzies 1988), humans (Bramley and Menzies 1988) and rat (Zailong and Stocco 2005). These data provide further support for a PR functionally distinct from the classic nuclear PR with predominant localization in an intracellular membrane such as the endoplasmic reticulum.

The apparent dissociation constant of 122 nM derived from saturation experiments is well within the range of values reported for other membrane P4-binding sites, including those found in bovine CL [197 nM] (Rae, Menzies et al. 1998), rat liver [170 nM] (Haukkamaa 1976) porcine liver [11-286 nM] (Meyer, Schmid et al. 1996) and the rat brain [160 nM] (Tischkau and Ramirez 1993). Additionally, in a recent paper investigating the various receptors for P4 in rat CL, which contains the mPR$\alpha$ homolog, an apparent dissociation constant of 162 nM was reported (Cai and Stocco 2005). Despite the low affinity for the ovine mPR, given its expression in the CL, it is probable that luteal membrane P4-binding sites are completely saturated because of the high concentration of P4 present in the CL. Further, to date very little data are available pertaining to the binding characteristics of the mammalian mPR homologs. Information presented in this chapter coupled with the recent binding studies in membranes from rat CL (Cai and Stocco 2005) provide some of the first data describing the binding characteristics of the mammalian mPRs. However, the binding data from rat mPR were obtained from membrane fractions of CL in which these researchers found expression of five genes previously postulated to encode for putative mPRs. The current ovine mPR$\alpha$ binding data are the first to demonstrate and characterize progestin binding in cells expressing the mPR$\alpha$ homolog alone. The low affinity for the ovine mPR plus the data demonstrating the lack of RU486 binding provide further evidence that the mammalian mPRs appear to function in a manner distinct from the nPRs.

# CHAPTER V.

## LIGAND ACTIVATION OF OVINE mPR CAUSES AN INCREASE IN INTRACELLULAR CALCIUM AND ACTIVATION OF JNK1/2

Introduction: In the previous chapter we demonstrated that progestins appear to be the ligand for the ovine mPR. However, to further verify that the ovine mPR is a *bona fide* receptor a functional response was needed. Numerous nongenomic actions have been reported for P4 as reviewed by Edwards (Edwards 2005). Nongenomic effects of P4 have been described in oocytes where P4 acts at the plasma membrane causing oocytes to progress to meiosis II (Godeau, Schorderet-Slatkine et al. 1978). Specifically, P4 inhibits adenylate cyclase causing a reduction in intracellular cAMP (Finidori-Lepicard, Schorderet-Slatkine et al. 1981; Sadler and Maller 1981). The reduction in cAMP translates into a decrease in the activity of PKA (Maller 1977). In human sperm, P4 and its metabolite 17$\alpha$-hydroxyprogesterone induce a rapid, and long-lasting influx of calcium ($Ca^{2+}$) (Thomas and Meizel 1989; Blackmore, Beebe et al. 1990; Blackmore and Lattanzio 1991), ultimately resulting in the acrosome reaction (Osman, Andria et al. 1989; Baldi, Casano et al. 1991). In the hypothalamus, P4 rapidly inhibits the frequency of GnRH release and consequently decreases the release of LH from the pituitary gland (Goodman, Bittman et al. 1981; O'Byrne, Thalabard et al. 1991). Nongenomic effects of acute exposure to P4 in the uterus include inhibition of transmembrane $Ca^{2+}$ entry, release of $Ca^{2+}$ from intracellular stores (Kosterin, Burdyga et al. 1994) and membrane

hyperpolarization with subsequent activation of $K^+$ channels (Mironneau, Savineau et al. 1981). Given the unique localization of the ovine mPR in the endoplasmic reticulum, we hypothesized that ligand stimulation of this receptor would increase intracellular $Ca^{2+}$ mobilization. As such, we wanted to determine if the ovine mPR alters intracellular concentrations of $Ca^{2+}$ after addition of progestins. Further, recent studies have demonstrated that the fish mPR$\alpha$ can promote phosphorylation of ERK1/2 (Zhu, Rice et al. 2003) and the human homolog can activate p38 MAKP (Karteris, Zervou et al. 2006). Thus, we also wanted to determine if the ovine mPR activates other intracellular pathways.

Materials and Methods

Measurement of Intracellular $Ca^{2+}$. Intracellular $Ca^{2+}$ was measured as described previously (Shlykov and Sanborn 2004). Briefly, CHO cells were plated in complete medium onto tissue culture dishes containing a #0 coverslip and the following day they were transfected with a mammalian expression vector encoding mPR (pcDNA3.1+) or control vector. At 24-48 h post-transfection, cells were loaded with Fura-2-AM (5 μM) (Molecular Probes, Eugene, OR) at room temperature for 30-35 min in fluorescence buffer (145 mM NaCl, 5mM KCl, 1 mM $Na_2HPO_4$, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 5 mM glucose, pH 7.4). After loading, cells were washed twice with fluorescence buffer and used after 35-45 min. For the experiments with extracellular $Ca^{2+}$ present, $Ca^{2+}$ concentrations were measured in fluorescence buffer. For the $Ca^{2+}$-free experiments (extracellular $Ca^{2+}$ absent), immediately prior to measuring $Ca^{2+}$,

fluorescence buffer was removed and replaced with $Ca^{2+}$-free fluorescence buffer supplemented with EGTA (100 μM). Progesterone (0.1-100 nM) or 17α-hydroxyprogesterone (100 nM) was added to cells and changes in free intracellular $[Ca^{2+}]_i$ in individual cells was measured at 340 and 380 nm excitation and 510 nm emission wavelengths with an InCyt2 imaging system (Intracellular Imaging Inc., Cincinnati, OH). To verify specificity of the ovine mPR for the progestins tested, cells expressing the ovine mPR were also treated with testosterone (100 nM), estradiol (100 nM), cortisol (100 nM) or RU486 (1-100 nM) and changes in free intracellular $[Ca^{2+}]_i$ were measured in individual cells. To determine if the released $Ca^{2+}$ was from the endoplasmic reticulum, cells were incubated with thapsigargin (100 nM) in $Ca^{2+}$-free buffer, to deplete $Ca^{2+}$ stores in the endoplasmic reticulum before treatment with progesterone or 17α-hydroxyprogesterone. In each dish, 40-80 individual cells were examined. Where indicated, the response per dish was averaged and data expressed as the mean 340/380 ratio ± S.E.. for the average values for *n* dishes. Statistical analysis was performed using the Newman-Keuls Multiple Comparison Test in Prism (Version 4a, from GraphPad Software, Inc.) and significance was taken as a value of $P < 0.05$. When variances were not homogenous, data were transformed by Log 10 function.

Western Blot Analysis. MCF7 cells were cultured in complete medium (RPMI 1640, supplemented with 10% FBS and penicillin-streptomycin (100 I.U. penicillin and 100 μg/mL streptomycin)) and were transfected with the encoding vector (pcDNA3.1+) for ovine mPR using the Polyfect procedure (QIAGEN, Valencia, CA). At 24-36h post-transfection, complete medium was removed and MCF7 cells transfected with ovine mPR

and non-transfected cells were cultured overnight in serum-free media prior to drug treatment and lysis. Cells were treated with nothing, vehicle alone (ethanol) or P4 (100 nM) for 20 min before lysis. To investigate if the ovine mPR influences a PLC pathway, additional experiments were conducted employing U73122 (Sigma, St. Louis, MO), a phospholipase C and $A_2$ inhibitor which inhibits the hydrolysis of PPI to $IP_3$. For a negative control in these experiments, a second set of cells expressing mPR were treated with U73343 (Sigma, St. Louis, MO), an inactive analog of U73122. For these experiments, cells were pretreated with U73122 or U73343 (5 $\mu$M) for 5 min before addition of P4 (100 nM) for 20 min. Following treatment, cells were washed with ice-cold PBS and lysed in radio-immunoprecipitation assay (RIPA) buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% NP40. 0.1% SDS, 0.5% deoxycholate, 2mM EDTA, 5mM sodium metavanadate, 5mM benzamidine and 1 mM phenylmethylsulfonyl fluoride (PMSF) on ice. The cell lysates were collected by centrifugation at 5000 x g for 10 min.

For analysis of p-JNK1/2 and JNK1/2, equal amounts of lysate from each fraction were separated using denaturing PAGE followed by transfer to nitrocellulose membranes for electroblotting. Membranes were blocked in TBS-T (140 mM NaCl, 10 mM Tris, 0.1% Tween 20, pH 7.4) + 5% non-fat milk for 1h at room temperature. For the analyses of phosphorylated JNK, membranes were incubated overnight at 4°C on an orbital shaker with an antibody (1:1000 dilution) specific for phospho-JNK obtained from Affinity Bioreagents (Golden, CO). For analysis of total JNK similar methods were performed except blots were incubated with an antibody (1:1000 dilution) that detects relative

amounts of JNK1/2 protein independent of phosphorylation (Cell Signaling Technology, Beverly, MA). Blots were then washed for 20-30 min with TBS-T before incubating for 1 h in 1% milk made in TBS-T with anti-rabbit IgG-HRP (1:2000 dilution) antibody. Again membranes were washed for 20-30 min and proteins were visualized by chemiluminescence (SuperSignal West Femto Maximum Sensitivity Substrate; Pierce, Rockford, IL).

Results: Changes in intracellular calcium $[Ca^{2+}]_i$ were measured in CHO cells transfected with ovine mPR or control vector (pcDNA3.1+). Initial studies were performed in a $Ca^{2+}$-containing buffer and P4 (100 nM) stimulated an increase in intracellular concentrations of $Ca^{2+}$, but only in CHO cells that expressed the ovine mPR (Fig 5.1). Figure 5.2 shows a representative tracing of the results obtained in CHO cells transfected with ovine mPR after addition of P4 (1 nM) in $Ca^{2+}$-free medium. Similar results were obtained after addition of 17α-hydroxyprogesterone (100 nM) to CHO cells expressing mPR in $Ca^{2+}$-free medium (Fig 5.3). Both progestins elicited a significant ($P < 0.05$) increase in $[Ca^{2+}]_i$, whereas there was no significant increase in $[Ca^{2+}]_i$ in cells transfected with control vector (Fig 5.4). As the increase in $[Ca^{2+}]_i$ occurred between 30 sec to 2 min post-progestin treatment with negligible changes in cells transfected with control vector, an average of the 340/380 ratio was determined for both mPR transfected and cells transfected with control vector from time of treatment to 5 min post treatment. As shown in Figure 5.4, the progestin-induced increase in $[Ca^{2+}]_i$ was absent in CHO cells transfected with control vector. Likewise, there was not an increase in concentrations of

$Ca^{2+}$ upon treatment with vehicle (EtOH, final concentration < 0.02%) (Fig 5.4). Only in cells expressing ovine mPR and treated with P4 (1 nM) or 17α-hydroxyprogesterone (100 nM) was there a significant ($P < 0.05$) increase in $[Ca^{2+}]_i$. Similar results were obtained in cells expressing the mPR-GFP construct (Fig 5.5). To verify if the increase in $[Ca^{2+}]_i$ was specific to the progestins, CHO cells transfected with ovine mPR were also treated with testosterone (100 nM), estradiol (100 nM), cortisol (100 nM), or RU486 (1-100 nM) in $Ca^{2+}$-free medium. No increase in $[Ca^{2+}]_i$ was detected after addition of testosterone, estradiol, cortisol or RU486 (Fig 5.6).

Given the unique localization of the ovine mPR, a second series of experiments was conducted to determine if an intracellular store of $Ca^{2+}$ was involved. To determine if the rise in $[Ca^{2+}]_i$ was due to $Ca^{2+}$ stored in the endoplasmic reticulum, cells transfected with ovine mPR were placed in a $Ca^{2+}$-free medium and first treated with thapsigargin (100 nM), an inhibitor of the $Ca^{2+}$ pump in the endoplasmic reticulum (Jackson, Patterson et al. 1988; Thastrup, Cullen et al. 1990). As shown in Figure 5.7, pretreatment with thapsigargin depleted the stores of intracellular $Ca^{2+}$ from the endoplasmic reticulum as indicated by the tracing and totally abolished the increase in $[Ca^{2+}]_i$ induced by P4 or 17α-hydroxyprogesterone.

The mPR first isolated was from spotted seatrout and was shown to phoshphorylate ERK1/2 (Zhu, Rice et al. 2003). However, treatment of cells expressing the ovine mPR with P4 did not increase phosphorylation of ERK1/2 (data not shown). Additionally, a recent paper reported that treatment of human myometrial cells with P4 results in activation of p38 MAPK (Karteris, Zervou et al. 2006). Similar to the ERK1/2

phosphorylation studies in cells expressing ovine mPR, ligand stimulation did not increase phosphorylation of p38 MAPK (data not shown).

As very little is currently known about the mammalian mPRs, studies were designed to investigate another intracellular MAPK pathway for the ovine mPR, specifically activation of stress-activated protein kinase/Jun-N-terminal kinase (SAPK/JNK). Treatment of non-transfected MCF7 cells with P4 did not increase the phosphorylation of JNK1/2, whereas treatment of MCF7 cells expressing ovine mPR with P4 for 20 min caused phosphorylation of JNK1/2 whereas there was little change in mPR transfected cells treated with vehicle or nothing (Fig 5.8). Total amounts of JNK were unchanged in these experiments (Fig 5.8). A potent and specific inhibitor of PLC, U73122 was used to determine if the ovine mPR possibly functions through a PLC mediated pathway. Pretreatment of MCF7 cells expressing ovine mPR with U73122 drastically decreased the phosphorylation of JNK1/2 by P4 (Fig 5.9). However, pretreatment with U73343, the inactive analog of U73122 did not alter the phosphorylation of JNK1/2 by P4 in MCF7 cells transfected with ovine mPR (Fig 5.9). Once again, total amounts of JNK1/2 were unchanged (Fig 5.9).

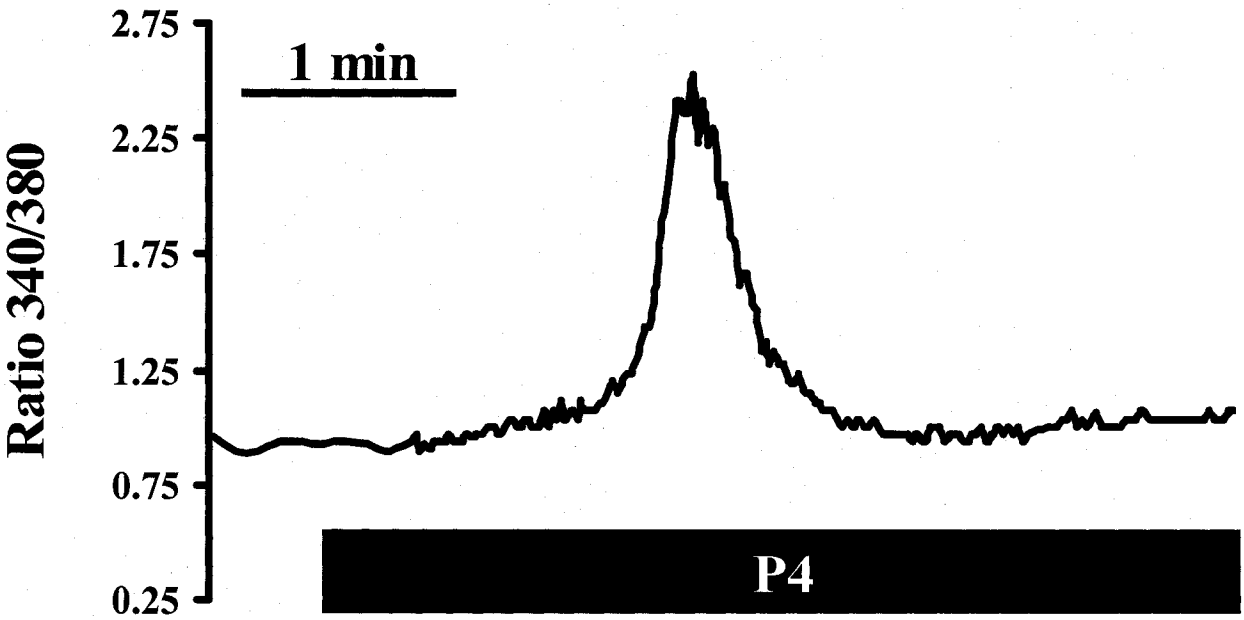


Figure 5.1. Effects of progesterone (P4) (100 nM) on $[Ca^{2+}]_i$ in CHO cells expressing ovine mPR. This is a representative tracing from a minimum of three experiments.

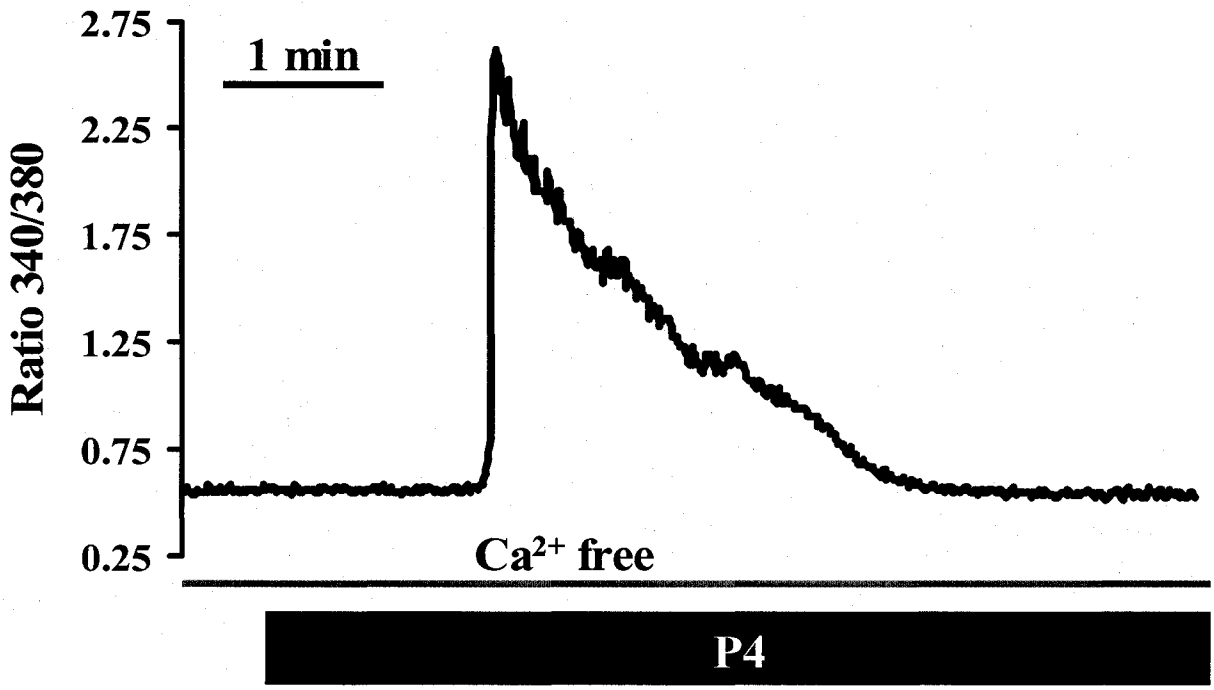


Figure 5.2. Effects of progesterone (P4) (1 nM) on $[Ca^{2+}]_i$ in CHO cells expressing ovine mPR. The tracing is representative from a minimum of three experiments in $Ca^{2+}$-free medium.

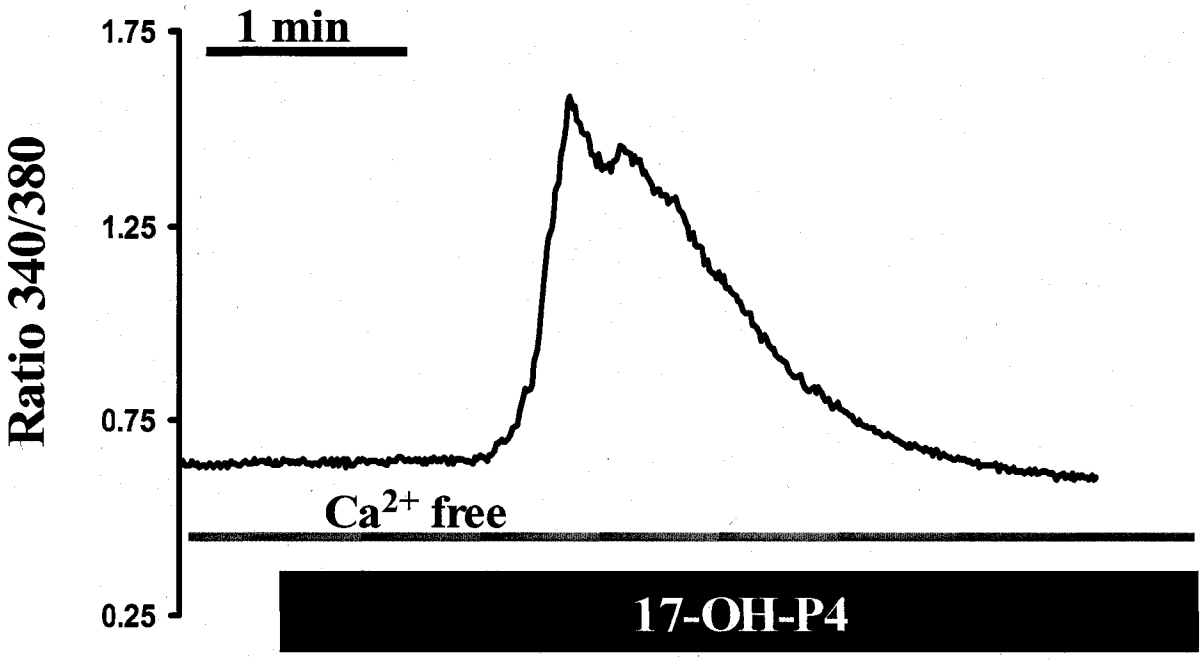


Figure 5.3. Effects of 17α-hydroxyprogesterone (17-OH-P4) (100 nM) on $[Ca^{2+}]_i$ in CHO cells expressing ovine mPR. The tracing is representative from a minimum of three experiments in $Ca^{2+}$-free medium.

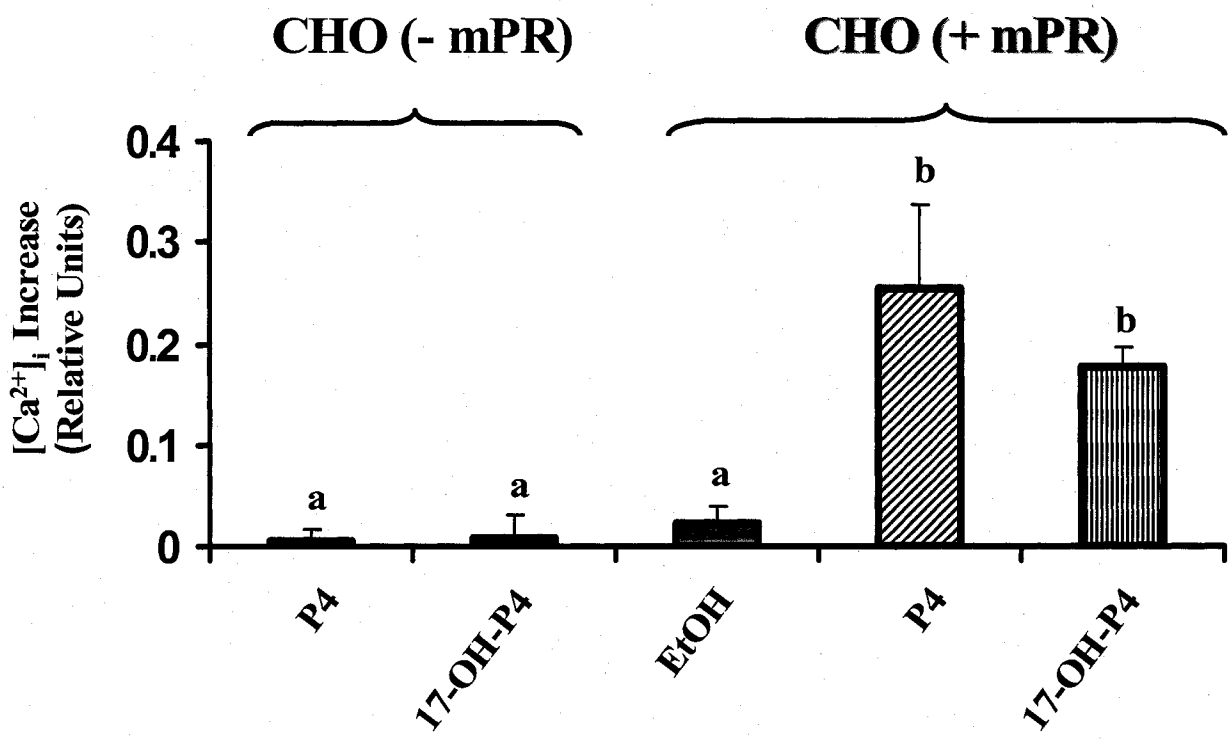


Figure 5.4. Change in net 340/380 ratios expressed in relative units of CHO cells transfected with ovine mPR or empty vector over 5 min period after treatment with progesterone (P4) (1 nM), 17α-hydroxyprogesterone (17-OH-P4) (100 nM), or EtOH. Significant differences ($P < 0.05$) between groups are indicated by different letters above the bars.

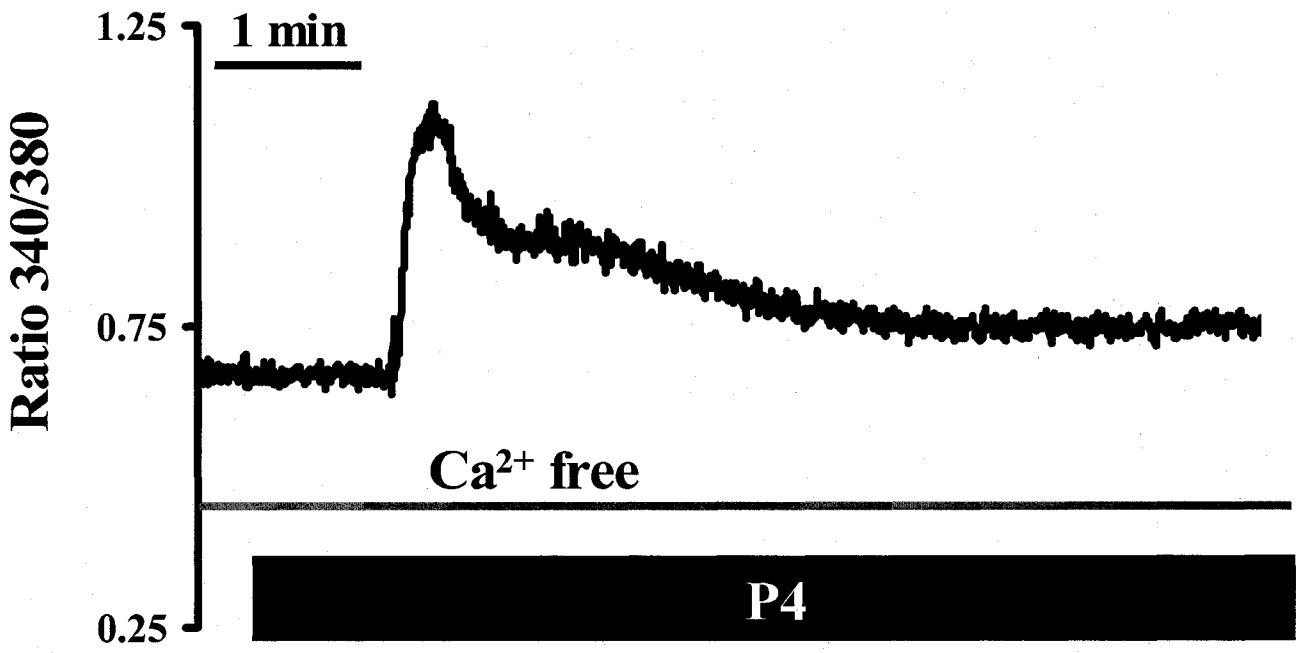


Figure 5.5. Effects of progesterone (P4) (1 nM) on $[Ca^{2+}]_i$ in CHO cells expressing ovine mPR-GFP construct. This is a representative tracing from a minimum of three experiments in $Ca^{2+}$-free medium.

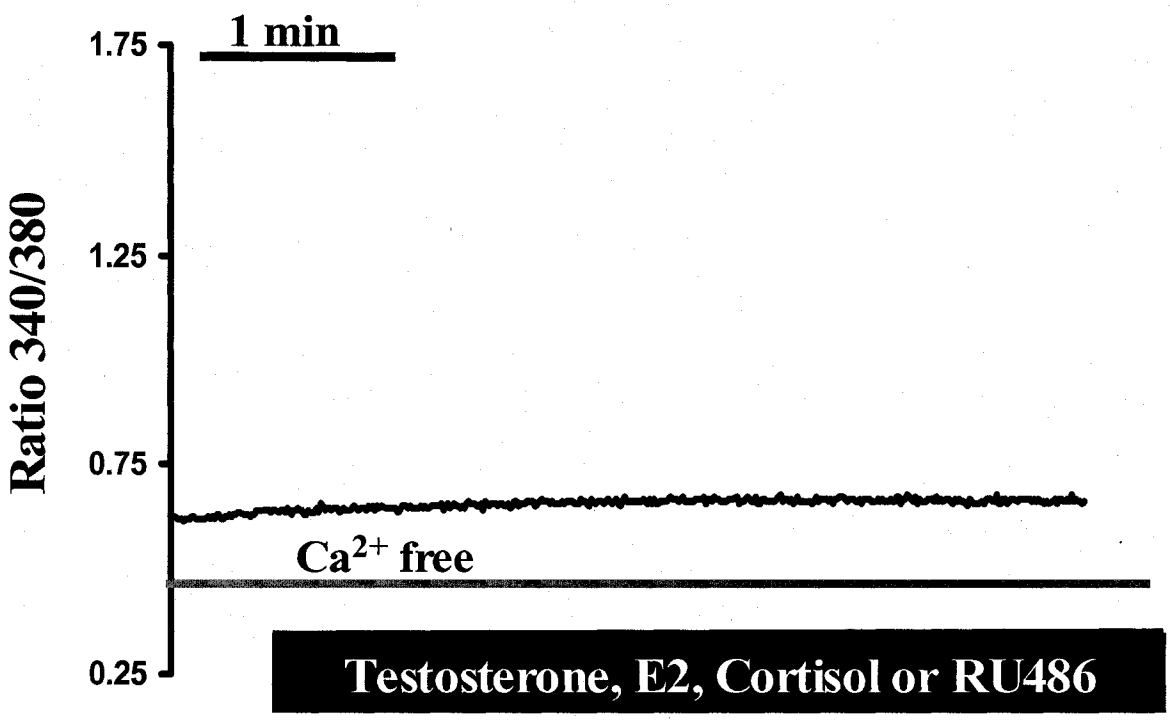


Figure 5.6. Effects of Testosterone (100 nM), Estradiol (E2),(100 nM), Cortisol (100 nM), or RU486 (100 nM) on $[Ca^{2+}]_i$ in CHO cells expressing ovine mPR. This is a representative tracing from a minimum of twelve experiments (3 for each treatment) in $Ca^{2+}$-free medium.

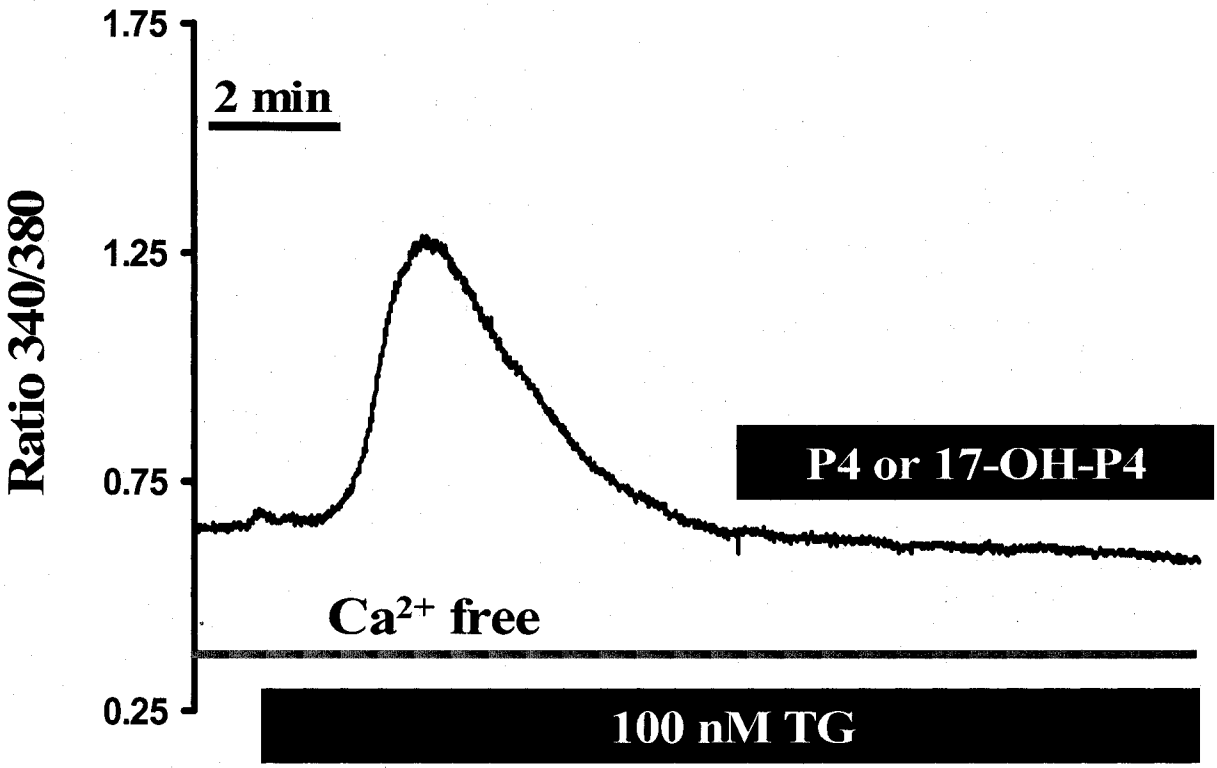


Figure 5.7. Effects of progesterone (P4) (1 nM) or 17α-hydroxyprogesterone (17-OH-P4) (100 nM) on $[Ca^{2+}]_i$ in CHO cells expressing ovine mPR were absent when cells were pretreated with thapsigargin (TG) (100 nM). Treatment with TG depleted the stores of $Ca^{2+}$ from the endoplasmic reticulum as indicated by the tracing. Progestins were added once cells returned to baseline. The tracing is a representative from a minimum of six experiments in $Ca^{2+}$-free medium.

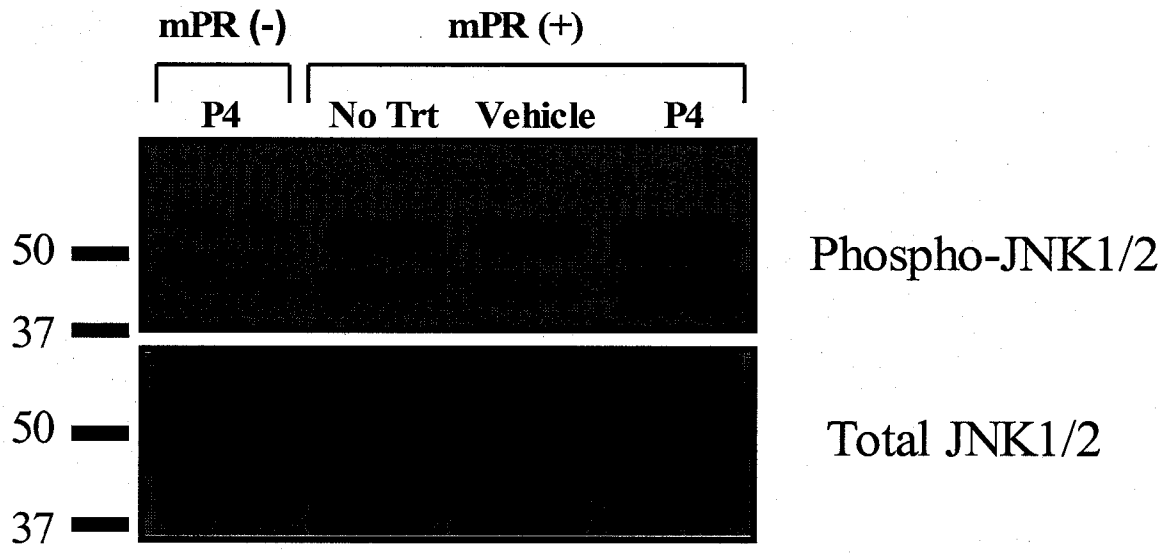


Figure 5.8. Analysis of phospho-JNK1/2 and total JNK1/2 by Western blot in nontransfected MCF7 cells and cells expressing ovine mPR. Cells were treated with nothing, vehicle, or P4 (100 nM) for 20 min before lysis. Equal amounts of each fraction were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with an anti-phospho-JNK1/2 antibody or total JNK1/2 antibody.

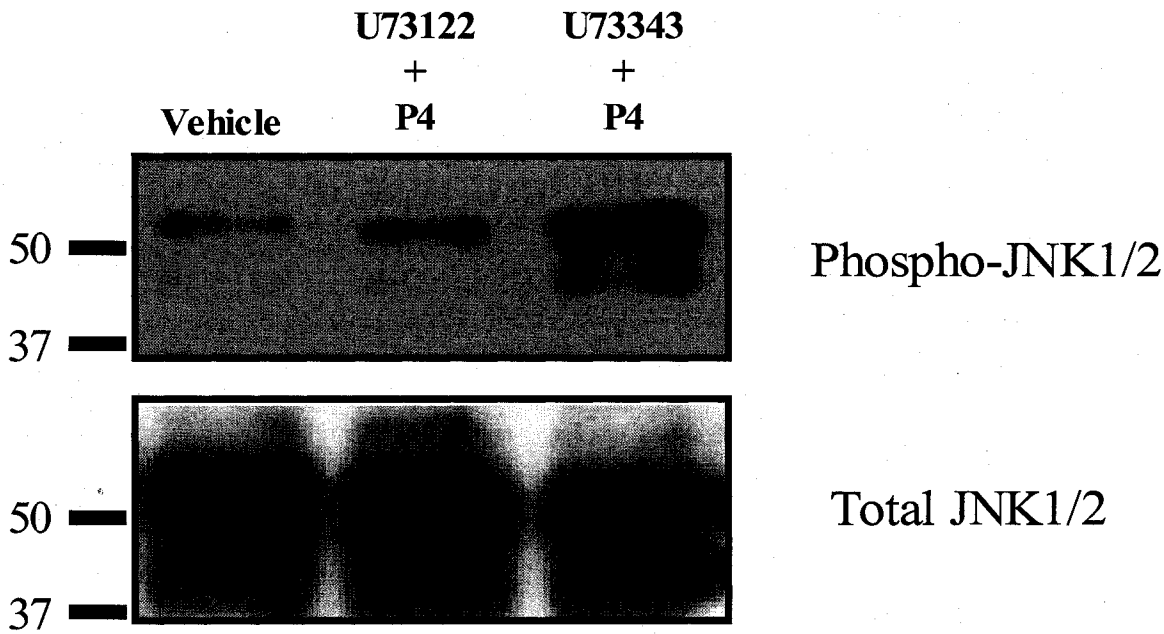


Figure 5.9. Analysis of phospho-JNK1/2 and total JNK1/2 by Western blot in MCF7 cells expressing ovine mPR. Cells were pretreated with nothing, U73122 (5 μM), or U73343 (5 μM) for 5 min. Next cells were treated with vehicle or P4 (100 nM) for 20 min before lysis. Equal amounts of each fraction were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with an antibody specific for phospho-JNK1/2 or for total JNK1/2.

Discussion: To date, mPR$\alpha$ has been studied most extensively for its role in mediating P4-induced maturation of ovaries in nonmammalian systems. Specifically, in seatrout ligand activation of mPR$\alpha$ increased MAPK activation within 5 min of treatment and also caused a significant reduction in production of cAMP (Zhu, Rice et al. 2003). Limited data exist for the possible role(s) of a mammalian mPR. Despite the data presented in this dissertation, the only other functional data to date for the mammalian mPRs was recently demonstrated in human myometrial cells (Karteris, Zervou et al. 2006). These researchers showed that activation of the mPRs ($\alpha$ and $\beta$) resulted in a decline in concentrations of cAMP and increased phosphorylation of myosin light chains, both of which are necessary for myometrial contraction.

Given the unique localization of ovine mPR in the endoplasmic reticulum as described in Chapter 3, we hypothesized that ligand stimulation of this receptor would increase mobilization of intracellular $Ca^{2+}$. As such, the objective of these studies was to determine if progestin activation of the ovine mPR alters concentrations of intracellular $Ca^{2+}$. The data presented in this chapter provide evidence that ligand activation of the ovine mPR leads to an increase in intracellular $Ca^{2+}$. Initial studies were performed in CHO cells transfected with the ovine mPR or control vector in the presence of extracellular $Ca^{2+}$. The only increase in intracellular $Ca^{2+}$ was observed in CHO cells expressing the mPR. To determine if the rise in intracellular $Ca^{2+}$ was due to an intracellular pool of $Ca^{2+}$, similar studies were conducted, but experiments were performed in $Ca^{2+}$-free medium. There was a rapid increase in concentrations of free intracellular $Ca^{2+}$ ($P < 0.05$) after addition of P4 (1-100 nM) or 17$\alpha$-hydroxyprogesterone

(100 nM) to CHO cells expressing ovine mPR. Treatment with progestins did not increase intracellular $Ca^{2+}$ in CHO cells transfected with control vector. Since these experiments were conducted in $Ca^{2+}$-free medium, the rise in intracellular $Ca^{2+}$ was believed to originate from the endoplasmic reticulum. To substantiate this hypothesis, cells were treated with thapsigargin to deplete $Ca^{2+}$ stores from the endoplasmic reticulum. Addition of progestins after treatment with thapsigargin to CHO cells expressing ovine mPR did not result in an increase in intracellular $Ca^{2+}$. This suggests that progestins act at the endoplasmic reticulum and is consistent with localization of mPR within the endoplasmic reticulum. The increase in intracellular $Ca^{2+}$ appears to be specific to progestins since treatment with estradiol, testosterone, cortisol, or RU486 did not evoke an increase in intracellular $Ca^{2+}$ in CHO cells transfected with mPR. Likewise, treatment with progestins did not stimulate an increase in concentrations of intracellular $Ca^{2+}$ in CHO cells transfected with control vector.

The existence of a functional intracellular transmembrane receptor does not fit with the common dogma of transmembrane receptors, which are thought to reside only in the plasma membrane. A functional intracellular receptor requires ligand passage across the plasma membrane and since P4 can easily cross the plasma membrane, it seems quite conceivable a functional mPR may be located in the endoplasmic reticulum. In support of this notion are the Ca2+ studies employing CHO cells expressing the ovine mPR-GFP construct. Treatment of these cells with P4 in a $Ca^{2+}$-free medium caused an increase in concentrations of intracellular $Ca^{2+}$. Interestingly, this is one of the constructs employed in the cellular localization studies in which mPR was predominately found in the

endoplasmic reticulum. As such, despite the GFP tag on the mPR, it still appears to function similarly to the native ovine mPR, further suggesting an action of progestins at the endoplasmic reticulum. Similar results have been described in studies using luteinized porcine granulosa cells, wherein P4 caused an increase in intracellular concentrations of $Ca^{2+}$ via $Ca^{2+}$ mobilization from the endoplasmic reticulum (Machelon, Nome et al. 1996). It was also demonstrated that the increase in $Ca^{2+}$ was a result of activation of phospholipase C linked to a pertussis-insensitive G-protein. This further suggests that a GPCR, specifically of the $G_q$ family is responsible for the increase in concentrations of $Ca^{2+}$ upon activation by P4 (Machelon, Nome et al. 1996). Interestingly, the nuclear PR antagonist, RU-38486 did not inhibit the P4-induced increase in $[Ca^{2+}]_i$ in luteinized porcine granulosa cells, suggesting the mPR has a different specificity than the classic nuclear PR (Machelon, Nome et al. 1996). Similarly, we did not observe an increase in $[Ca^{2+}]_i$ after treatment with RU486 (1-100 nM) in CHO cells transfected with the ovine mPR. Since the ovine mPR is distinct from the nuclear PRs and is expressed in luteal cells, it will be intriguing to determine whether the ovine mPR works through a pathway similar to luteinized porcine granulosa cells to cause $Ca^{2+}$ mobilization.

The current findings that ligand activation of the ovine mPR leads to JNK activation are also interesting. As mentioned, very little data are available pertaining to possible functions for the mammalian mPRs. However, both the fish mPR$\alpha$ and human homolog activate various portions of the MAPK pathway. In spotted seatrout, mPR$\alpha$ activated ERK1/2 (Zhu, Rice et al. 2003) whereas the human mPR$\alpha$ causes

phosphorylation of p-38 (Karteris, Zervou et al. 2006). The ovine mPRα similar to the human homolog does not appear to phosphorylate ERK1/2 (Karteris, Zervou et al. 2006). It is also interesting that an inhibitor of PLC (U73122) blocked activation of JNK1/2 by P4 whereas the inactive analog (U73343) did not. From these data, it appears the mPRα functions through a PLC mediated pathway.

The existence of a transmembrane intracellular receptor for P4 is very intriguing, as it goes against the common dogma that transmembrane receptors reside only in the plasma membrane. Further, the data presented herein provide evidence for an intracellular receptor that activates the MAPK pathway, which again has often been thought to occur only through activation of receptors at the plasma membrane. However, as reviewed by Mor and Philips (Mor and Philips 2006), in addition to the plasma membrane, Ras and /or MAPK signaling has now been observed on endosomes, the endoplasmic reticulum, the Golgi apparatus, and mitochondria. Specific to the endoplasmic reticulum, Chiu and coworkers demonstrated that when oncogenic Ras was targeted to the endoplasmic reticulum with a transmembrane tether, it retained full transforming activity. This indicates that all the signaling events required for the complex cellular phenotype of transformation can be set into motion from internal membranes (Chiu, Bivona et al. 2002). These researchers also demonstrated that the endoplasmic reticulum-tethered Ras was a potent activator of JNK (Chiu, Bivona et al. 2002). As such, it seems conceivable that the ovine mPRα also functions through a PLC-mediated pathway, specifically at the endoplasmic reticulum, and upon ligand activation causes activation of JNK1/2. This new concept of subcellular compartmentalization of

signaling adds another level of regulation that may permit greater specificity and segregation of signal outputs. Elucidation of these complex signaling networks in a physiological context may represent a new frontier in signal transduction research.

Despite the functional data for the ovine mPR presented in this chapter, knowledge of its overall functional role is uncertain. It is tempting to speculate that the ovine mPR is possibly involved in apoptosis, given that ligand activation of this receptor leads to increased intracellular $Ca^{2+}$ and phosphorylation of JNK1/2, both of which are involved in apoptosis (Davis 2000). Further, the ovine mPR is expressed in luteal tissue and apoptosis is involved in structural regression of the CL (McGuire, Juengel et al. 1994; Rueda, Wegner et al. 1995). It seems plausible the ovine mPR may have some functional role in regulating lifespan of the CL. Moreover, it is interesting to note that treatment of bovine luteal cells with the nPR antagonist, RU486, increased apoptosis in these cells. This suggests a role for the nPR in maintaining the CL (Rueda, Hendry et al. 2000). Given that the ovine mPR does not appear to bind RU486, as described in chapter 4, it seems possible that there is intracellular communication between the nPRs and the mPR to regulate lifespan of the CL. In support of these interactions, it was recently shown in human myometrial cells that the human mPR interacts with the nPR (Karteris, Zervou et al. 2006). Using antibodies specific for mPR and nPR-B followed by immunofluorescent staining, Karteris and coworkers demonstrated colocalization of the two proteins. Whether the ovine nPR and mPR interact is currently not known.

# CHAPTER VI

# CONCLUSIONS

The studies herein describe the cloning and characterization of two ovine membrane P4 receptors, PGRMC1 and mPRα. Both proteins are distinct from the nuclear P4 receptors and based on the data in this dissertation, both appear to be intracellular membrane P4 receptors. To date, no other laboratory has reported the existence of these two proteins in sheep.

According to current literature, the first protein described in this dissertation, PGRMC1, is localized within the plasma membrane of rat granulosa cells (Peluso, Pappalardo et al. 2006), cytosol of rat granulosa and luteal cells (Peluso, Pappalardo et al. 2006), and the endoplasmic reticulum of porcine liver (Falkenstein, Meyer et al. 1996; Meyer, Schmid et al. 1996; Selmin, Lucier et al. 1996). However, we are the first to demonstrate specific localization of this protein to the endoplasmic reticulum using PGRMC1-GFP in combination with a marker (pdsRed2-ER) specific for the endoplasmic reticulum. PGRMC1 has been coined a receptor by other laboratories (Falkenstein, Meyer et al. 1996; Meyer, Schmid et al. 1996), yet in our hands the ovine PGRMC1 does not behave in a similar fashion compared to the porcine PGRMC1. First, incubation of crude membrane fractions from CHO cells expressing ovine PGRMC1 with $^{3}$H-P4 did not result in any specific binding. Despite employing methods similar to Falkenstein and colleagues, who demonstrated binding of $^{3}$H-P4 in membrane fractions from CHO cells expressing the porcine PGRMC1, the ovine PGRMC1 does not appear to bind P4

(Falkenstein, Heck et al. 1999). Second, the ovine PGRMC1 is not involved in mediating $Ca^{2+}$ mobilization. In human sperm the P4-induced $Ca^{2+}$ increase was reduced in the presence of an antibody against the porcine PGRMC1 (Falkenstein, Heck et al. 1999). We have demonstrated that in CHO cells expressing PGRMC1, treatment with P4 does not increase concentrations of intracellular $Ca^{2+}$. It is important to note that the data described in this dissertation do not exclude the possibility that PGRMC1 is involved in mediating actions of P4 or that in ovine sperm PGRMC1 may function differently than in CHO cells. However the endpoints assayed herein do not support a direct role for PGRMC1 causing an increase in intracellular $Ca^{2+}$ in response to P4. In rat granulosa cells, PGRMC1 forms a complex with PAIRBP1, and this complex appears to function in the antiapoptotic action of P4 (Peluso, Pappalardo et al. 2006). As such, the ovine PGRMC1 may interact with other proteins not examined by the experiments described herein, to participate in cellular signaling in response to P4.

We are the first to demonstrate the existence of the second protein, mPR, in the sheep. Further, we have provided the first characterization of mPR expression throughout the estrous cycle of the sheep, in the hypothalamus, pituitary, uterus, ovary, and CL. To date, changes in expression of mPR have been demonstrated only in the rat CL during pregnancy (Cai and Stocco 2005) and in human myometrial cells following exposure to P4 or estradiol (Karteris, Zervou et al. 2006). We are the first to describe the *in vivo* profile for changes in expression of mPR in the hypothalamus, pituitary and ovary for any mammalian species during the estrous cycle, and the first to report these changes in

the uterus and CL in sheep. Characterizing fluctuations in expression may aid in elucidation of the physiological functions of mPR.

Additionally, a unique feature of mPR that we first described is its localization to the endoplasmic reticulum rather than the plasma membrane. Since mPR contains seven hydrophobic amino acid domains indicative of a prototypical GPCR, plasma membrane localization was anticipated. Thus its localization only in the endoplasmic reticulum was somewhat surprising. Further, our report of mPR localization in the endoplasmic reticulum (Ashley, Clay et al. 2006) has since been corroborated in cells expressing the human mPR (Krietsch, Fernandes et al. 2006).

We are the first to demonstrate binding of $^{3}$H-P4 in membrane fractions from cells expressing ovine mPR. Currently, limited binding data exists for the mPR, and reports published have utilized either recombinant mPR or membrane fractions from rat CL. Specificity of ligand binding is similar between the ovine mPR and other mPRs in the literature from seatrout, human, and rat (Zhu, Bond et al. 2003; Zhu, Rice et al. 2003; Cai and Stocco 2005) in which P4, 17$\alpha$-hydroxyprogesterone, and 20$\alpha$-hydroxyprogesterone appear to be ligands for mPR. The ovine mPR shares greater similarities with the rat mPR than with the nonmammalian homolog. The $K_d$ reported in membrane fractions from rat CL is 162 nM which is similar to the 122 nM $K_d$ for the ovine mPR. It is noteworthy to mention that the rat CL expresses 5 genes which encode for putative membrane P4 receptors (Cai and Stocco 2005). Thus, direct comparison of our data with theirs is difficult, because it is not known which of the 5 putative membrane P4 receptors is binding with P4 in membrane fractions from rat CL.

Progesterone induces $Ca^{2+}$ mobilization in sperm (Thomas and Meizel 1988; Krausz, Bonaccorsi et al. 1995) and granulosa cells (Machelon, Nome et al. 1996; Younglai, Wu et al. 2005), yet the receptor perpetuating this physiological response is not known. We provide evidence that the ovine mPR directly induces $Ca^{2+}$ release from the endoplasmic reticulum upon ligand activation. This was demonstrated in cells expressing the native protein as well as in cells expressing the mPR-GFP construct, in which predominant localization of mPR in the endoplasmic reticulum was first observed. Further, the ovine mPR appears to activate the MAPK pathway, specifically phosphorylation of JNK1/2 upon ligand activation.

The studies presented in this dissertation provide exciting new information to direct future research in the field of reproductive physiology. Past studies have reported binding of P4 within microsomal fractions from corpora lutea from a variety of mammalian species, (Bramley and Menzies 1988; Bramley and Menzies 1988; Bramley and Menzies 1988; Bramley and Menzies 1993; Menzies and Bramley 1994), however, the protein interacting with P4 was not elucidated. The ovine mPR is the first mammalian protein identified that both localizes to the endoplasmic reticulum and binds P4. Additionally, we have demonstrated the expression of mPR in the hypothalamus, pituitary, uterus, ovary, and CL, tissues known to respond in a nongenomic manner to P4. While further studies are required to characterize the actions of mPR in these tissues, the expression of this atypical P4 receptor provides an exciting venue for research into mediation of nongenomic effects of P4.

To date there is little information regarding the function(s) of the mammalian mPR. Studies described in this dissertation provide the first functional roles for the ovine mPR. Data involving action of P4 at the hypothalamus and/or pituitary is scarce, but it is known that P4 decreases the frequency of GnRH pulses and consequently the release of LH (Goodman, Bittman et al. 1981; O'Byrne, Thalabard et al. 1991), processes which require $Ca^{2+}$ mobilization to function properly. Compromising any interpretation however, is the presence of nPRs in the hypothalamus (Skinner, Caraty et al. 2001) and pituitary (Tasende, Rodriguez-Pinon et al. 2005). As yet, it is undetermined if physiological concentrations of P4 interact with one or all receptors for P4 (membrane and nuclear) in these tissues. If P4 binds both types of receptors, the protein actually involved in the overall biological response needs to be determined. To address this issue, studies need to be completed in which one of the receptors has been eliminated to delineate the specific functions of each receptor. Since ovine mPR causes an increase in concentrations of intracellular $Ca^{2+}$ upon ligand activation it is possible that mPR may be involved in the release of GnRH or LH and that the nPRs are involved in negative feedback. Thus, the final biological result probably depends on the cross-talk between the nPRs and mPR and the summation of the intracellular events associated with each receptor.

As early as 1981, Rothchild proposed that P4 has an intraovarian site of action (Rothchild 1981). Since this report many researchers have investigated the effects of P4 on the ovary and CL and demonstrated that P4 plays a major role in mitosis and apoptosis in granulosa and luteal cells (Peluso 2006), as well as in regulating its own synthesis

(Rothchild 1996). The data from this dissertation provides strong evidence that the ovine mPR causes an increase in concentrations of intracellular $Ca^{2+}$ upon ligand activation. It is possible that P4 may regulate its own production through the generation of this $Ca^{2+}$ signal since $Ca^{2+}$ is involved in regulating steroidogenesis (Flores and Veldhuis 1993). Further, expression of the ovine mPR in the CL appears to be regulated in a manner that coincides with the capacity of the CL to synthesize P4. Comparing serum concentrations of P4 to the expression of mPR in the CL, it is evident that expression of mPR closely mimics serum concentrations of P4 suggesting that the ovine mPR is possibly regulated by P4 and that the mPR plays a central role in the regulation of CL function. Additionally, we have demonstrated that activation of ovine mPR causes phosphorylation of JNK1/2. Since both $Ca^{2+}$ and the JNK pathway are involved in apoptosis (Davis 2000), which is essential to structural regression of the CL (McGuire, Juengel et al. 1994; Rueda, Wegner et al. 1995), it is possible that activation of the ovine mPR may lead to apoptosis of luteal cells. Thus, the ovine mPR may have some functional role in regulating lifespan of the CL. Moreover, treatment of bovine luteal cells with the nPR antagonist, RU486, increases apoptosis in these cells, suggesting a role for the nPR in maintaining the CL (Rueda, Hendry et al. 2000). Given that the ovine mPR does not bind RU486 as described in chapter 4, it seems possible that intracellular communication between the nPRs and the mPR may be involved in regulation of lifespan of the CL.

It is important to appreciate that none of these intracellular events associated with P4 are mutually exclusive. For example, if one considers the ovine CL, not only are the nPRs expressed, but also PGRMC1 and mPR, all of which reportedly respond to P4 and

the possibility exists that other proteins will be identified that may bind P4 and initiate intracellular events as well. In addition to the CL, every tissue in which the ovine mPR is expressed as described in this dissertation, also expresses nPRs. As a consequence, attempting to decipher which protein is responsible for each biological action attributed to P4 becomes extremely difficult. In all likelihood, the overall physiological response of P4 within a particular tissue is due to: 1) the expression pattern of these putative membrane P4 receptors and nPRs, 2) the binding affinity of P4 for each of these receptor systems, 3) the concentration of P4 available at a specific time, and 4) the communication between these receptor systems. As such, it is the overall temporal/spatial expression of these membrane receptors for P4 coupled with availability of ligand that determines the final biological response for each tissue. Moreover, further research is needed to determine if there is intracellular communication between these membrane receptors for P4 and the nPRs and how they elicit physiological functions. Given that each of these complex receptor systems appears to bind P4 or P4 metabolites, albeit at different affinities, the possibility exist that progestin specific drugs could be developed to specifically treat various reproductive cancers and disorders.

In summary, the groundwork and a firm basis for future studies directed at identifying the unique biological roles of the ovine mPR have been established. The existence of a unique form of P4 receptor that contains seven transmembrane domains and resides in the endoplasmic reticulum is exciting and fuels enthusiasm for further research into the mode of action for this receptor. This novel method of signaling at the endoplasmic reticulum adds to the intricacy of signaling in cells and provides a

mechanistically unique method for initiating actions of progesterone that may alter classical steroid dogma regarding the mechanisms by which steroid hormones act.

## CHAPTER VII

## LITERATURE CITED


Adams, T. E., T. O. Wagner, et al. (1979). "GnRH interaction with anterior pituitary. II. Cyclic AMP as an intracellular mediator in the GnRH activated gonadotroph." Biol Reprod **21**(3): 735-47.

Ahmed, C. E. and G. D. Niswender (1981). "Internalization and degradation of human chorionic gonadotropin in ovine luteal cells: effects of inhibitors of transglutaminase." Endocrinology **109**(5): 1388-93.

Ashley, R. L., C. M. Clay, et al. (2006). "Cloning and characterization of an ovine intracellular seven transmembrane receptor for progesterone that mediates calcium mobilization." Endocrinology **147**(9): 4151-9.

Baldi, E., R. Casano, et al. (1991). "Intracellular calcium accumulation and responsiveness to progesterone in capacitating human spermatozoa." J Androl **12**(5): 323-30.

Baulieu, E. E. and M. Schumacher (2000). "Progesterone as a neuroactive neurosteroid, with special reference to the effect of progesterone on myelination." Hum Reprod **15 Suppl 1**: 1-13.

Beato, M. (1989). "Gene regulation by steroid hormones." Cell **56**: 335-344.

Bernauer, S., M. Wehling, et al. (2001). "The human membrane progesterone receptor gene: Genomic structure and promoter analysis." DNA Sequence **12**: 13-25.

Bielefeldt, K., L. Waite, et al. (1996). "Nongenomic effects of progesterone on human intestinal smooth muscle cells." Am J Physiol **271**(2 Pt 1): G370-6.

Blackmore, P. F., S. J. Beebe, et al. (1990). "Progesterone and 17 alpha-hydroxyprogesterone. Novel stimulators of calcium influx in human sperm." J Biol Chem **265**(3): 1376-80.

Blackmore, P. F. and F. A. Lattanzio (1991). "Cell surface localization of a novel non-genomic progesterone receptor on the head of human sperm." Biochem Biophys Res Commun **181**(1): 331-6.

Bogacki, M., W. J. Silvia, et al. (2002). "Direct inhibitory effect of progesterone on oxytocin-induced secretion of prostaglandin F(2alpha) from bovine endometrial tissue." Biol Reprod **67**(1): 184-8.

Boonyaratanakornkit, V., M. Scott, et al. (2001). "Progesterone receptor contains a proline-rich motif that directly interacts with SH3 domains and activates c-Src family tyrosine kinases." Molecular Cell **8**: 269-280.

Bramley, T. A. and G. S. Menzies (1988). "Association of progesterone with a unique particulate fraction of the human corpus luteum." J Endocrinol **116**: 307-312.

Bramley, T. A. and G. S. Menzies (1988). "Subcellular fractionation of the ovine corpus luteum: association of progesterone with ovine luteal membranes?" Mol Cell Endocrinol **59**(1-2): 135-46.

Bramley, T. A. and G. S. Menzies (1988). "Subcellular fractionation of the porcine corpus luteum: sequestration of progesterone in a unique particulate fraction." J Endocrinol **117**(3): 341-54.

Bramley, T. A. and G. S. Menzies (1993). "Specificity studies of particulate binding sites for steroid hormones in subcellular fractions of the porcine corpus luteum." J Endocrinol **136**(3): 371-80.
Bramley, T. A. and G. S. Menzies (1994). "Particulate binding sites for steroid hormones in subcellular fractions of the ovine corpus luteum: properties and hormone specificity." Mol Cell Endocrinol **103**(1-2): 39-48.
Bu, S. Z., D. L. Yin, et al. (1997). "Progesterone induces apoptosis and up-regulation of p53 expression in human ovarian carcinoma cell lines." Cancer **79**(10): 1944-50.
Cai, Z. and C. Stocco (2005). "Expression and regulation of progestin membrane receptors in the rat corpus luteum." Endocrinology **146**(12): 5522-5532.
Chiu, V. K., T. Bivona, et al. (2002). "Ras signalling on the endoplasmic reticulum and the Golgi." Nat Cell Biol **4**(5): 343-50.
Conneely, O. M. and J. P. Lydon (2000). "Progesterone receptors in reproduction: functional impact of the A and B isoforms." Steroids **65**(10-11): 571-7.
Conneely, O. M., B. Mulac-Jericevic, et al. (2003). "Progesterone-dependent regulation of female reproductive activity by two distinct progesterone receptor isoforms." Steroids **68**(10-13): 771-8.
Davis, R. J. (2000). "Signal transduction by the JNK group of MAP kinases." Cell **103**(2): 239-52.
DeBold, J. F. and C. A. Frye (1994). "Genomic and non-genomic actions of progesterone in the control of female hamster sexual behavior." Horm Behav **28**(4): 445-53.
Drouva, S. V., E. Laplante, et al. (1985). "Progesterone-induced LHRH release in vitro is an estrogen--as well as Ca++- and calmodulin-dependent secretory process." Neuroendocrinology **40**(4): 325-31.
Duckworth, B. C., J. S. Weaver, et al. (2002). "G2 arrest in Xenopus oocytes depends on phosphorylation of cdc25 by protein kinase A." Proc Natl Acad Sci U S A **99**(26): 16794-9.
Due, W., K. P. Dieckmann, et al. (1989). "Immunohistological determination of oestrogen receptor, progesterone receptor, and intermediate filaments in Leydig cell tumours, Leydig cell hyperplasia, and normal Leydig cells of the human testis." J Pathol **157**(3): 225-34.
Duleba, A. J., R. Z. Spaczynski, et al. (1999). "Divergent mechanisms regulate proliferation/survival and steroidogenesis of theca-interstitial cells." Mol Hum Reprod **5**(3): 193-8.
Dunlap, K. A. and F. Stormshak (2004). "Nongenomic inhibition of oxytocin binding by progesterone in the ovine uterus." Biol Reprod **70**(1): 65-9.
Edwards, D. P. (2005). "Regulation of signal transduction pathways by estrogen and progesterone." Annu Rev Physiol **67**: 335-76.
Ehring, G. R., H. H. Kerschbaum, et al. (1998). "A nongenomic mechanism for progesterone-mediated immunosuppression: inhibition of K+ channels, Ca2+ signaling, and gene expression in T lymphocytes." J Exp Med **188**(9): 1593-602.
Everett, J. (1948). "Progesterone and estrogen in the experimental control of ovulation time and other features of estrous cycle in the rat." Endocrinology **43**: 389-405.

Falkenstein, E., M. Heck, et al. (1999). "Specific progesterone binding to a membrane protein and related nongenomic effects on Ca2+-fluxes in sperm." Endocrinology **140**(12): 5999-6002.

Falkenstein, E., C. Meyer, et al. (1996). "Full-length cDNA sequence of a progesterone membrane-binding protein from porcine vascular smooth muscle cells." Biochem Biophys Res Commun **229**(1): 86-9.

Falkenstein, E., K. Schmieding, et al. (1998). "Localization of a putative progesterone membrane binding protein in porcine hepatocytes." Cell Mol Biol (Noisy-le-grand) **44**(4): 571-8.

Finidori-Lepicard, J., S. Schorderet-Slatkine, et al. (1981). "Progesterone inhibits membrane-bound adenylate cyclase in Xenopus laevis oocytes." Nature **292**(5820): 255-7.

Fitz, T. A., M. H. Mayan, et al. (1982). "Characterization of two steroidogenic cell types in the ovine corpus luteum." Biol Reprod **27**(3): 703-11.

Flores, J. and J. Veldhuis (1993). "Single cell studies of calcium second messenger signaling pathway in ovarian granulosa cells." In: Adashi EY, Leung PCK (eds), "The Ovary." New York: Raven;: 129-145.

Foresta, C., M. Rossato, et al. (1992). "Progesterone induces capacitation in human spermatozoa." Andrologia **24**(1): 33-5.

Formby, B. and T. S. Wiley (1999). "Bcl-2, survivin and variant CD44 v7-v10 are downregulated and p53 is upregulated in breast cancer cells by progesterone: inhibition of cell growth and induction of apoptosis." Mol Cell Biochem **202**(1-2): 53-61.

Freedman, L. P. (1992). "Anatomy of the steroid receptor zinc finger region." Endocr Rev **13**(2): 129-45.

Frye, C. A., L. E. Bayon, et al. (2000). "Intravenous progesterone elicits a more rapid induction of lordosis in rats than does SKF38393." Psychobiology **28**: 99-109.

Glusa, E., T. Graser, et al. (1997). "Mechanisms of relaxation of rat aorta in response to progesterone and synthetic progestins." Maturitas **28**(2): 181-91.

Godeau, J. F., S. Schorderet-Slatkine, et al. (1978). "Induction of maturation in Xenopus laevis oocytes by a steroid linked to a polymer." Proc Natl Acad Sci U S A **75**: 2353-2357.

Goodman, R. L., E. L. Bittman, et al. (1981). "The endocrine basis of the synergistic suppression of luteinizing hormone by estradiol and progesterone." Endocrinology **109**(5): 1414-7.

Gore-Langton, P. and D. Armstrong (1988). "Follicular Steroidogenesis and its Control. In: Knobil E, Neill J (eds.), The Physiology of Reproduction, New York: Raven Press;." **1**: 331-387.

Grazzini, E., G. Guillon, et al. (1998). "Inhibition of oxytocin receptor function by direct binding of progesterone." Nature **392**(6675): 509-12.

Hanekamp, E. E., S. C. Gielen, et al. (2003). "Progesterone receptors in endometrial cancer invasion and metastasis: development of a mouse model." Steroids **68**(10-13): 795-800.

Harrison, D. A., D. W. Carr, et al. (2000). "Involvement of protein kinase A and A kinase anchoring protein in the progesterone-initiated human sperm acrosome reaction." Biol Reprod **62**(3): 811-20.
Haukkamaa, M. (1976). "Progesterone-binding properties of microsomes from rat liver." J Steroid Biochem **7**(5): 345-50.
Hirata, S., T. Shoda, et al. (2000). "The novel isoform of the progesterone receptor cDNA in the human testis and detection of its mRNA in the human uterine endometrium." Oncology **59 Suppl 1**: 39-44.
Ismail, P. M., P. Amato, et al. (2003). "Progesterone involvement in breast development and tumorigenesis--as revealed by progesterone receptor "knockout" and "knockin" mouse models." Steroids **68**(10-13): 779-87.
Jackson, T. R., S. I. Patterson, et al. (1988). "A novel tumor promoter, thapsigargin, transiently increases cytoplasmic free calcium without generation of inositol phoshphates in NG 115-401 L neural cells." Biochem J **253**: 81-86.
Jiang, C. W., P. M. Sarrel, et al. (1992). "Progesterone induces endothelium-independent relaxation of rabbit coronary artery in vitro." Eur J Pharmacol **211**(2): 163-7.
Jiang, H., K. M. Whitworth, et al. (2004). "Large-scale generation and analysis of expressed sequence tags from porcine ovary." Biol Reprod **71**(6): 1991-2002.
Kalra, S. P. (1993). "Mandatory neuropeptide-steroid signaling for the preovulatory luteinizing hormone-releasing hormone discharge." Endocr Rev **14**(5): 507-38.
Karteris, E., S. Zervou, et al. (2006). "Progesterone signaling in human myometrium through two novel membrane G protein-coupled receptors: potential role in functional progesterone withdrawal at term." Mol Endocrinol **20**(7): 1519-34.
Kastner, P., A. Krust, et al. (1990). "Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B." Embo J **9**(5): 1603-14.
Ke, F. C. and V. D. Ramirez (1987). "Membrane mechanism mediates progesterone stimulatory effect on LHRH release from superfused rat hypothalami in vitro." Neuroendocrinology **45**(6): 514-7.
Key, T. J. (1995). "Hormones and cancer in humans." Mutat Res **333**(1-2): 59-67.
Kosterin, S. A., T. V. Burdyga, et al. (1994). Mechanisms of calcium transport in myometrium. Control of Uterine Contractility. R. E. G. a. T. N. Tabb. Boca Raton, FL, CRC: 129-154.
Krausz, C., L. Bonaccorsi, et al. (1995). "Intracellular calcium increase and acrosome reaction in response to progesterone in human spermatozoa are correlated with in-vitro fertilization." Hum Reprod **10**(1): 120-4.
Krietsch, T., M. S. Fernandes, et al. (2006). "Human Homologs of the Putative G Protein-Coupled Membrane Progestin Receptors (mPR{alpha}, {beta}, {gamma}) Localize to the Endoplasmic Reticulum and are Not Activated by Progesterone." Mol Endocrinol.
Kubli-Garfias, C. and R. E. Whalen (1977). "Induction of lordosis behavior in female rats by intravenous administration of progestins." Horm Behav **9**(3): 380-6.
Leonhardt, S., V. Boonyaratanakornkit, et al. (2003). "Progesterone receptor transcription and non-transcription signaling mechanisms." Steroids. **68**(10-31): 761-70.

Leonhardt SA, Boonyaratanakornkit V, et al. (2003). "Progesterone receptor transcription and non-transcription signaling mechanisms." Steroids. **68**(10-31): 761-70.
Lisk, R. D. (1960). "A comparison of the effectiveness of intravenous, as opposed to subcutaneous, injection of progesterone for the induction of estrous behavior in the rat." Can J Med Sci **38**: 1381-3.
Losel, R., S. Breiter, et al. (2005). "Classic and non-classic progesterone receptors are both expressed in human spermatozoa." Horm Metab Res **37**(1): 10-4.
Losel, R., A. Dorn-Beineke, et al. (2004). "Porcine spermatozoa contain more than one membrane progesterone receptor." Int J Biochem Cell Biol **36**(8): 1532-41.
Lutz, L. B., B. Kim, et al. (2000). "G protein beta gamma subunits inhibit nongenomic progesterone-induced signaling and maturation in Xenopus laevis oocytes. Evidence for a release of inhibition mechanism for cell cycle progression." J Biol Chem **275**(52): 41512-20.
Machelon, V., F. Nome, et al. (1996). "Progesterone triggers rapid transmembrane calcium influx and/or calcium mobilization from endoplasmic reticulum, via a pertussis-insensitive G-protein in granulosa cells in relation to luteinization process." J Cell Biochem **61**(4): 619-28.
Maller, J. L. (2001). "The elusive progesterone receptor in Xenopus oocytes." Proc Natl Acad Sci U S A **98**(1): 8-10.
Maller, J. L. a. K., E.G. (1977). "Progesterone-stimulated meiotic cell division in Xenopus oocytes. Induction by regulatory subunit and inhibition by catalytic subunit of adenosine 3`:5`-monophosphate-dependent protein kinase." J. Biol. Chem **252**: 1712-1718.
McCarthy, M. M., E. Felzenberg, et al. (1995). "Infusions of diazepam and allopregnanolone into the midbrain central gray facilitate open-field behavior and sexual receptivity in female rats." Horm Behav **29**(3): 279-95.
McGuire, W. J., J. L. Juengel, et al. (1994). "Protein kinase C second messenger system mediates the antisteroidogenic effects of prostaglandin F2 alpha in the ovine corpus luteum in vivo." Biol Reprod **51**(4): 800-6.
McRae, R. S., H. M. Johnston, et al. (2005). "Changes in mouse granulosa cell gene expression during early luteinization." Endocrinology **146**(1): 309-17.
Meizel, S., K. O. Turner, et al. (1997). "Progesterone triggers a wave of increased free calcium during the human sperm acrosome reaction." Dev Biol **182**(1): 67-75.
Menzies, G. S. and T. A. Bramley (1994). "Specific binding sites for progesterone in subcellular fractions of the porcine corpus luteum." J Endocrinol **142**(1): 101-10.
Meyer, C., R. Schmid, et al. (1996). "Purification and partial sequencing of high-affinity progesterone-binding site(s) from porcine liver membranes." Eur J Biochem **239**(3): 726-31.
Meyerson, B. (1972). "Latency between intravenous injection of progestins and the appearance of estrous behavior in estrogen-treated ovariectomized rats." Horm Behav **3**(1): 1-9.
Migliaccio, A., D. Piccolo, et al. (1998). "Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor." Embo J **17**(7): 2008-18.

Minshall, R. D., D. Pavcnik, et al. (2002). "Nongenomic vasodilator action of progesterone on primate coronary arteries." J Appl Physiol **92**(2): 701-8.
Mironneau, J., J. P. Savineau, et al. (1981). "Fast outward current controlling electrical activity in rat uterine smooth muscle during gestation." J Physiol (Paris) **77**(8): 851-9.
Mor, A. and M. R. Philips (2006). "Compartmentalized Ras/MAPK signaling." Annu Rev Immunol **24**: 771-800.
Moreau, M., J. P. Vilain, et al. (1980). "Free calcium changes associated with hormone action in amphibian oocytes." Dev Biol **78**(1): 201-14.
Nemere, I., R. J. Pietras, et al. (2003). "Membrane receptors for steroid hormones: signal transduction and physiological significance." J Cell Biochem **88**(3): 438-45.
Niswender, G. D. (1973). "Influence of the site of conjugation on the specificity of antibodies to progesterone." Steroids **22**(3): 413-24.
Niswender, G. D. and T. M. Nett (1988). "The Corpus Luteum and its Control." In: Knobil E, Neill J (eds), The Physiology of Reproduction. New York: Raven; **1**: 489-526.
O'Byrne, K. T., J. C. Thalabard, et al. (1991). "Radiotelemetric monitoring of hypothalamic gonadotropin-releasing hormone pulse generator activity throughout the menstrual cycle of the rhesus monkey." Endocrinology **129**(3): 1207-14.
Osman, R. A., M. L. Andria, et al. (1989). "Steroid induced exocytosis: the human sperm acrosome reaction." Biochem Biophys Res Commun **160**(2): 828-33.
Peluso, J. J. (2006). "Multiplicity of progesterone's actions and receptors in the mammalian ovary." Biol Reprod **75**(1): 2-8.
Peluso, J. J., A. Pappalardo, et al. (2006). "Progesterone membrane receptor component 1 expression in the immature rat ovary and its role in mediating progesterone's antiapoptotic action." Endocrinology **147**(6): 3133-40.
Pfaff, D. W. (1997). "Hormones, genes, and behavior." Proc Natl Acad Sci U S A **94**(26): 14213-6.
Pfaff, D. W., S. Schwartz-Giblin, et al. (1994). Cellular and molecular mechanisms of female reproductive behaviors. The physiology of reproduction. E. Knobil and J. Neil. New York, Raven Press: 107-219.
Pfaffl, M. W. (2001). "A new mathematical model for relative quantification in real-time RT-PCR." Nucleic Acids Res **29**(9): e45.
Rae, M. T., G. S. Menzies, et al. (1998). "Specific non-genomic, membrane-localized binding sites for progesterone in the bovine corpus luteum." Biol Reprod **58**(6): 1394-406.
Reilander, H., C. Reinhart, et al. (2000). G-Protein-Coupled Receptors. Boca Raton, CRC.
Revankar, C. M., D. F. Cimino, et al. (2005). "A transmembrane intracellular estrogen receptor mediates rapid cell signaling." Science **307**: 1625-1630.
Richer, J. K., B. M. Jacobsen, et al. (2002). "Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells." J Biol Chem **277**(7): 5209-18.

Roesch, D. M. and M. Keller-Wood (1997). "Progesterone rapidly reduces arterial pressure in ewes." Am J Physiol **272**(1 Pt 2): H386-91.
Rose, J. D. (1990). Brainstem influences on sexual behavior. Brainstem Mechanisms of Behavior. W. R. Klemm and R. P. Vertes. Wiley, NY: 407-463.
Rossato, M., A. Nogara, et al. (1999). "Identification of functional binding sites for progesterone in rat Leydig cell plasma membrane." Steroids **64**(1-2): 168-75.
Rothchild, I. (1981). "The regulation of the mammalian corpus luteum." Recent Prog Horm Res **37**: 183-298.
Rothchild, I. (1996). "The corpus luteum revisited: are the paradoxical effects of RU486 a clue to how progesterone stimulates its own secretion?" Biol Reprod **55**(1): 1-4.
Rueda, B. R., I. R. Hendry, et al. (2000). "Decreased progesterone levels and progesterone receptor antagonists promote apoptotic cell death in bovine luteal cells." Biol Reprod **62**(2): 269-76.
Rueda, B. R., J. A. Wegner, et al. (1995). "Internucleosomal DNA fragmentation in ovine luteal tissue associated with luteolysis: in vivo and in vitro analyses." Biol Reprod **52**(2): 305-12.
Sabeur, K., D. P. Edwards, et al. (1996). "Human sperm plasma membrane progesterone receptor(s) and the acrosome reaction." Biol Reprod **54**(5): 993-1001.
Sadler, S. E. and J. L. Maller (1981). "Progesterone inhibits adenylate cyclase in Xenopus oocytes. Action on the guanine nucleotide regulatory protein." J Biol Chem **256**(12): 6368-73.
Saner, K. J., B. H. Welter, et al. (2003). "Cloning and expression of a novel, truncated, progesterone receptor." Mol Cell Endocrinol **200**(1-2): 155-63.
Sasson, R., E. Rimon, et al. (2004). "Gonadotrophin-induced gene regulation in human granulosa cells obtained from IVF patients. Modulation of steroidogenic genes, cytoskeletal genes and genes coding for apoptotic signalling and protein kinases." Mol Hum Reprod **10**(5): 299-311.
Selmin, O., G. W. Lucier, et al. (1996). "Isolation and characterization of a novel gene induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin in rat liver." Carcinogenesis **17**(12): 2609-15.
Selye, H. (1942). "Correlations between the chemical structure and the pharmacological actions of the steroids." Endocrinology **30**: 437-453.
Senger, P. L. (1999). Pathways to Pregnancy and Parturition. Ephrata, PA, Current Conceptions, Inc.
Sheridan, P. J., J. L. Phillips, et al. (1975). "Modulation of the uptake and retention of estradiol-17beta in the ovine corpus luteum by luteinizing hormone." Proc Soc Exp Biol Med **149**(2): 537-40.
Shlykov, S. G. and B. M. Sanborn (2004). "Stimulation of intracellular calcium oscillations by diacylglycerol in human myometrial cells." Cell Calcium **36**: 157-164.
Skinner, D. C., A. Caraty, et al. (2001). "Unmasking the progesterone receptor in the preoptic area and hypothalamus of the ewe: no colocalization with gonadotropin-releasing neurons." Endocrinology **142**(2): 573-9.

Skinner, D. C., N. P. Evans, et al. (1998). "The negative feedback actions of progesterone on gonadotropin-releasing hormone secretion are transduced by the classical progesterone receptor." Proc Natl Acad Sci U S A **95**(18): 10978-83.
Soloff, M. S. and P. Sweet (1982). "Oxytocin inhibition of (Ca2+ + Mg2+)-ATPase activity in rat myometrial plasma membranes." J Biol Chem. **257**(18): 10687-93.
Soyal, S., P. M. Ismail, et al. (2002). "Progesterone's role in mammary gland development and tumorigenesis as disclosed by experimental mouse genetics." Breast Cancer Res **4**(5): 191-6.
Stein, D. G. (2001). "Brain damage, sex hormones and recovery: a new role for progesterone and estrogen?" Trends Neurosci **24**(7): 386-91.
Tasende, C., M. Rodriguez-Pinon, et al. (2005). "Corpus luteum life span and pituitary oestrogen and progesterone receptors in cyclic and gonadotrophin-releasing hormone-treated anoestrous ewes." Reprod Fertil Dev **17**(7): 721-6.
Tesarik, J., J. Moos, et al. (1993). "Stimulation of protein tyrosine phosphorylation by a progesterone receptor on the cell surface of human sperm." Endocrinology **133**(1): 328-35.
Thastrup, O., P. J. Cullen, et al. (1990). "Thapsigargin, a tumor promoter, discharges intracellular calcium stores by specific inhibtion of the endoplasmic reticulum calcium-ATPase." Proc Natl Acad Sci U S A **87**: 5466-2470.
Thomas, A. J., R. P. Nockels, et al. (1999). "Progesterone is neuroprotective after acute experimental spinal cord trauma in rats." Spine **24**(20): 2134-8.
Thomas, P. and S. Meizel (1988). "An influx of extracellular calcium is required for initiation of the human sperm acrosome reaction induced by human follicular fluid." Gamete Res **20**(4): 397-411.
Thomas, P. and S. Meizel (1989). "Phosphatidylinositol 4,5-bisphosphate hydrolysis in human sperm stimulated with follicular fluid or progesterone is dependent upon Ca2+ influx." Biochem J **264**(2): 539-46.
Tischkau, S. A. and V. D. Ramirez (1993). "A specific membrane binding protein for progesterone in rat brain: sex differences and induction by estrogen." Proc Natl Acad Sci U S A **90**(4): 1285-9.
Tsai, M. J. and B. W. O'Malley (1994). "Molecular mechanisms of action of steroid/thyroid receptor superfamily members." Annu Rev Biochem **63**: 451-86.
Vegeto, E., M. M. Shahbaz, et al. (1993). "Human progesterone receptor A form is a cell- and promoter-specific repressor of human progesterone receptor B function." Mol Endocrinol **7**(10): 1244-55.
Wasserman, W. J., L. H. Pinto, et al. (1980). "Progesterone induces a rapid increase in [Ca2+]in of Xenopus laevis oocytes." Proc Natl Acad Sci U S A **77**(3): 1534-6.
Wei, L. L., P. Hawkins, et al. (1996). "An amino-terminal truncated progesterone receptor isoform, PRc, enhances progestin-induced transcriptional activity." Mol Endocrinol **10**(11): 1379-87.
Wei, L. L., B. M. Norris, et al. (1997). "An N-terminally truncated third progesterone receptor protein, PR(C), forms heterodimers with PR(B) but interferes in PR(B)-DNA binding." J Steroid Biochem Mol Biol **62**(4): 287-97.

Younglai, E. V., Y. J. Wu, et al. (2005). "Non-genomic action of estradiol and progesterone on cytosolic calcium concentrations in primary cultures of human granulosa-lutein cells." Hum Reprod **20**(9): 2383-90.

Zailong, C. and C. Stocco (2005). "Expression and regulation of progestin membrane receptors in the rat corpus luteum." Endocrinology published August 25, 2005;10.1210/en.2005-0759.

Zhu, X. L., P. S. Sexton, et al. (2001). "Characterization of membrane steroid binding protein mRNA and protein in lens epithelial cells." Exp Eye Res **73**(2): 213-9.

Zhu, Y., J. Bond, et al. (2003). "Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor." Proc Natl Acad Sci U S A **100**(5): 2237-2242.

Zhu, Y., C. D. Rice, et al. (2003). "Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes." Proc Natl Acad Sci U S A **100**(5): 2231-6.