DISSERTATION

REGULATION OF STEROIDOGENIC ACUTE REGULATORY (StAR) PROTEIN AND EVIDENCE THAT CONSTITUTIVE STEROIDOGENESIS IN OVINE LARGE LUTEAL CELLS IS MEDIATED BY TONICALLY ACTIVE PROTEIN KINASE A

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY RANDY LLOYD BOGAN ENTITLED **REGULATION OF STEROIDOGENIC ACUTE REGULATORY (StAR) PROTEIN AND EVIDENCE THAT CONSTITUTIVE STEROIDOGENESIS IN OVINE LARGE LUTEAL CELLS IS MEDIATED BY TONICALLY ACTIVE PROTEIN KINASE A** BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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Abstract of Dissertation

REGULATION OF STEROIDOGENIC ACUTE REGULATORY (StAR) PROTEIN AND EVIDENCE THAT CONSTITUTIVE STEROIDOGENESIS IN OVINE LARGE LUTEAL CELLS IS MEDIATED BY TONICALLY ACTIVE PROTEIN KINASE A

Progesterone is required for maintenance of pregnancy in all-mammalian species and is produced by the corpus luteum (CL) during estrous/menstrual cycles and early pregnancy. The rate-limiting step in steroidogenesis and the step most acutely regulated by second messengers is the transport of cholesterol across the mitochondrial membrane, a process mediated by the steroidogenic acute regulatory protein (StAR). Factors activating the cyclic AMP (cAMP)/protein kinase-A (PKA) pathway stimulate steroidogenesis by increasing expression, and activity of StAR via phosphorylation. The CL contains two steroidogenic cell types; large cells which have high constitutive progesterone secretion, and small cells that have low basal progesterone production, which increases in response to stimulation by tropic hormones. To determine if tonically active PKA causes constitutive steroidogenesis by large cells, purified large and small ovine luteal cells were treated with a specific PKA inhibitor (PKI) and the adenylate cylase activator forskolin. Effects on progesterone and cAMP production, and the expression and phosphorylation state of StAR, were quantified. Additionally, the mechanism of StAR-associated cholesterol transport may involve the peripheral-type

benzodiazepine receptor (PBR). Bioluminescence resonance energy transfer (BRET) was used to test StAR/PBR interactions.

Treating small cells with PKI prevented forskolin-induced increases in progesterone secretion without affecting cAMP production. In large cells, basal secretion of progesterone and relative StAR phosphorylation were significantly decreased following PKI treatment, indicating the presence of tonically active PKA. There was a significant correlation between StAR phosphorylation and progesterone secretion, but no correlation between the quantity of StAR and secretion of progesterone. This indicates that phosphorylation, but not changes in quantity, is the primary effect of PKA to regulate StAR's activity. No StAR/PBR interactions were detected by BRET analysis, although data were obtained indicating that PBR aggregates in the mitochondrial membrane and may form a pore through which cholesterol can pass.

In summary, a novel assay for quantifying concentrations and relative phosphorylation states of StAR was developed and used to provide evidence that constitutive steroidogenesis in ovine large luteal cells is mediated by tonically active PKA. Additionally, it appears that PBR aggregates in the mitochondrial membrane, but no evidence was obtained that StAR and PBR interact.

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Introduction

Historical Background

The corpus luteum (CL) is a transient endocrine gland that originates from cells of the follicle following ovulation. The CL is the source of progesterone during estrous/menstrual cycles and early pregnancy, and therefore proper CL function is necessary to provide progesterone required for regulating estrous/menstrual cycle length and supporting a pregnancy if conception occurs. In 1903, Ludwig Fraenkel demonstrated that maintaining pregnancy is dependent on a substance secreted by the CL when he performed lutectomy on pregnant rabbits, which resulted in a termination of pregnancy (1, as cited in 2). Interestingly, when Fraenkel began these studies on the CL, the terms "hormone" and "endocrinology" were foreign to the scientific community (2). The chain of events that lead to Fraenkel's experiment began between 1895 and 1897 when the anatomist Johannes Sobotta published drawings of the mouse CL (2). These drawings included an adenoid structure and ductless gland. These anatomical structures within the CL convinced Sobotta's colleague, Gustav Jacob Born, that the CL functioned as a gland which secreted its products into the blood (2). Prior to this, Auguste Prenant hypothesized that the CL was a gland of internal secretion, but Prenant believed the secreted substance prevented ovulation during an existing pregnancy (2). Born explained

his hypothesis to Fraenkel who began the experiments on rabbits. Thirty-one years after Fraenkel's initial paper that proved the relationship between the CL and maintenance of pregnancy, four separate groups of scientists reported on the successful isolation of progesterone (3-6, as cited in 2). Although it is not known which group was the first to isolate progesterone, the group from Breslau, Germany was the first to publish the correct formula for progesterone (3, as cited in 2). Interestingly, the Breslau team was formed when Fraenkel recruited the help of chemist Karl Heinrich Slotta who was Fraenkel's son-in-law, along with Slotta's Ph.D. candidate Heinrich Ruschig and Fraenkel's assistant Erich Fels, to try and isolate the factor secreted by the CL that maintained pregnancy (2). Sadly, shortly following their accomplishment, Fraenkel and Fels who were Jews, along with Slotta who was married to Fraenkel's daughter, were forced into emigration due to political pressure from the ruling Nazi party in Germany (2). They were able to resume scientific careers in North and South America, but never continued their joint research on progesterone (2).

Biologic Effects of Progesterone

Progesterone is required for maintenance of pregnancy in all-mammalian species (7). Progesterone has a "calming" effect on the uterus that aids attachment of the conceptus. It inhibits estradiol-induced contractions of the myometrium by preventing α-adrenergic receptor expression, decreases extracellular calcium uptake, and modifies electrical coupling between myometrial cells, all of which are necessary for contraction to occur (reviewed in 8). Progesterone creates a proper uterine environment for embryo development prior to implantation by stimulating glandular secretions and modifying

proteins secreted by endometrial cells (8). Progesterone prevents ovulation via negative feedback on the hypothalamus and anterior pituitary. In the hypothalamus, progesterone prevents surges of gondadotropin-releasing hormone (GnRH) (reviewed in 8). In the anterior pituitary, progesterone decreases the number of GnRH receptors, inhibits expression of the common α-subunit of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and decreases transcription of the genes encoding the unique β-subunits of LH and FSH (9).

Formation of the Corpus Luteum

Luteinization

Luteinization, which is the differentiation of follicular cells into luteal cells following ovulation, is stimulated by the preovulatory gonadotropin surge (8, 10). Immediately following ovulation, the follicle is called a corpus hemorrhagicum because of its "blood clot"-like appearance (8), although ovulation itself is not prerequisite for luteinization to begin in all instances or species (reviewed in 10). The follicle loses its hemorrhagic appearance approximately 3-5 days post-ovulation as it rapidly increases in size and absorbs the clotted blood (8). The preovulatory follicle has four layers consisting of theca externa, theca interna, basement membrane, and granulosa (11). The preovulatory gonadotropin surge causes the basement membrane to disintegrate, which allows mixing of thecal and granulosal cells in most non-primate species (Reviewed in 12). In general, following follicular rupture and release of the ovum, the walls of the follicle "collapse" which causes the formation of many folds (11, 13). Invasion of thecal cells into the granulosa layer occurs at these folds as the basement membrane disintegrates, which

results in a mixing of theca, granulosa, and connective tissue cells (11, 13). Vascular tissues present at the folds infiltrate the center cavity, and at the same time strands of connective tissue are formed across the radius of the CL. The radial strands of connective tissue allow larger blood vessels to be integrated within the CL (10). The CL will become extensively vascularized (8), and receives a higher rate of blood flow than other tissue in the body (6-10 $ml \bullet g^{-1} \bullet min^{-1}$) (8, 14). This is necessary to meet the metabolic demands of the CL, demands that exceed the oxygen consumption per unit of weight when compared to the heart, kidney, or liver (8). The CL will rapidly increase in size as illustrated by an approximately 15-fold increase in weight in sheep compared to the follicle from which it originated (8). An extensive capillary network is formed which allows luteal cells to be positioned in close proximity to blood vessels or interstitial space (8), thus allowing rapid delivery of progesterone produced by the CL to the systemic circulation. It has been estimated that the human CL secretes 10-40 mg of progesterone per day (15).

Cellular Composition of the Corpus Luteum

There are two steroidogenic cell types in the CL, commonly referred to as small and large luteal cells. In sheep, small cells are designated as <22 μM in diameter while large cells are >22 μM (16). Small and large steroidogenic luteal cells have been described in pigs (17), ewes (16), cattle (18), rabbits (19), and humans (20). It is widely believed that small and large steroidogenic cells originate from thecal and granulosal cells respectively (reviewed in 10). One of the first descriptions of luteinization occurred in pigs, which included the conversion of granulosal cells to luteal cells, a controversial idea at the time (13, as cited in 10). In primates, the theca and granulosa layers do not intermix to the

same degree as in other species due to at least a partial maintenance of the basement membrane following ovulation (21). The two steroidogenic cell types in primates are called granulosa-lutein and theca-lutein cells based on their presumed origins, and they exhibit the same steroidogenic characteristics as large and small luteal cells respectively (reviewed in 8).

Rapid growth of the CL during the early luteal phase occurs by different mechanisms in each of the steroidogenic cell types. The granulosa-derived cells undergo hypertrophy, with anywhere from a 2-8 fold increase in volume, but the number of cells remain relatively constant as mitosis does not occur (8, 10). This increase in volume arises from an increase in the cytoplasmic:nuclear ratio, and an increase in many organelles associated with steroidogenesis (reviewed in 22). The number of granulosal cells in the preovulatory follicle closely approximates the number of large cells in the ovine CL (23), and monoclonal antibodies directed against granulosal cell-surface antigens preferentially bound to large luteal cells in developed bovine CL (24), providing evidence for the non-dividing nature and origin of large cells. Small cells increase in numbers up to 5-fold in sheep (8). Alkaline phosphatase is localized to the theca interna of the preovulatory follicle in sheep, and ovine small luteal cells have alkaline phosphatase activity thus providing correlative evidence of their origin from thecal cells (11). Large cells compose ~40% of CL volume, but only ~10% of the total cell number, while small cells make up ~20% of CL volume and 25% of the number of cells (reviewed in 14). In sheep, both large and small cells contain abundant smooth endoplasmic reticulum and mitochondria, consistent with a high steroidogenic capacity. Large cells contain secretory granules and

rough endoplasmic reticulum indicating they have protein-secreting capacity, while small cells lack both (14). One hormone identified within the secretory granules of large cells is oxytocin (25). The production and release of oxytocin by large cells is important for luteolysis to occur in most species.

There are approximately five different types of cells in the developed ovine CL (23). In addition to steroidogenic cells, endothelial cells, fibroblasts, pericytes, and cells from the bloodstream can account for ~50% of the volume of the CL and 60% of the number of cells (23). The number of fibroblasts doubles during the luteal phase of the ovine estrous cycle, and the number of endothelial cells increases ~6.5 fold (23). The proliferation of endothelial cells is important for vascularization of the CL (reviewed in 8, 14).

Progesterone Synthesis

Cholesterol Supply

The precursor for all steroid hormones is cholesterol. Luteal cells can obtain cholesterol by two mechanisms: 1.) uptake of cholesterol from the blood via low-density lipoprotein (LDL) or high-density lipoprotein (HDL) (28-30), and 2.) *de novo* synthesis of cholesterol from acetate (31-34). Either LDL or HDL, produced primarily in the liver, can be used as a cholesterol source, although there are differences between species in preference for LDL or HDL (reviewed in 35). In humans, LDL is the predominant lipoprotein and preferred cholesterol source (34). Human granulosal cells can efficiently use HDL as well (36), but there is still dissention on whether or not HDL is utilized by the human CL throughout the luteal phase (reviewed in 37). The uptake of LDL has been

well characterized. Receptors on the plasma membrane of luteal cells bind LDL, and the ligand/receptor complex is internalized by receptor-mediated endocytosis (38). The internalized endosome is incorporated into a lysosome. The interior of the lysosome is acidic which causes the LDL molecule to dissociate from its receptor, and the receptor is degraded or recycled back to the plasma membrane (reviewed in 35). Uptake of HDL also occurs via a membrane receptor; however, this does not appear to involve receptor-mediated endocytosis (reviewed in 39). Cholesterol intake from HDL involves a non-lysosomal (i.e. selective) pathway in which cholesterol esters from the HDL molecule are internalized without their corresponding apoprotein (36). The scavenger receptor type 1 class B (SR-B1) is an HDL receptor, and the mechanism of SR-B1 mediated cholesterol internalization has been detailed (40).

Lipoproteins are the primary source of cholesterol under normal physiologic conditions. Luteal cells appear to only use *de novo* synthesis under severely lipid-deprived states or under *in vitro* conditions (reviewed in 8). In support of this, steroidogenic tissues have low concentrations of HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, and relatively low levels of other enzymes involved in the cholesterol biosynthetic pathway, which indicates that *de novo* cholesterol synthesis only plays a minor role in normal steroidogenesis (34, 41). Free cholesterol within luteal cells can be stored as cholesterol esters via the action of cholesterol ester synthetase (8). These cholesterol esters may form lipid droplets, which are often noted as a morphologic characteristic of steroidogenic cell types (reviewed in 8).

Conversion of Cholesterol to Progesterone

Progesterone synthesis involves two enzymatic conversions of cholesterol. First, the side chain of the cholesterol molecule is removed by cytochrome p450 side-chain cleavage enzyme ($p450_{scc}$), located on the inner mitochondrial membrane, thus forming pregnenolone. Pregnenolone is converted to progesterone in the smooth endoplasmic reticulum by 3β-hydroxysteroid dehydrogenase/Δ5, Δ4 isomerase (3β-HSD) (reviewed in 26). Progesterone is the primary hormone secreted by the CL, but other steroids may be produced depending on species and/or reproductive state. Most notably, estrogens are synthesized by the primate CL during the luteal phase of the menstrual cycle (27).

Rate Limiting Step in Steroidogenesis

Given that progesterone synthesis involves only two enzymatic conversions by $p450_{scc}$ and 3β-HSD, it seems likely that a deficiency in progesterone secretion would be due to one or both enzymes' limiting the production of progesterone. However, during the majority of the luteal phase, neither enzyme is limiting to progesterone synthesis. Expression of $p450_{scc}$ in luteal tissue is high and held constant throughout the luteal phase of primates (42, 43), indicating that $p450_{scc}$ is not a primary point of regulation in progesterone biosynthesis. Treatment of purified large and small ovine luteal cells with hydroxylated cholesterol molecules, which can cross the mitochondrial membrane without the aid of transport proteins, was used to measure $p450_{scc}$ activity. These studies provided evidence that even with high concentrations of cholesterol at the inner mitochondrial membrane, $p450_{scc}$ activity is not limiting to progesterone synthesis (44). However, there is evidence that $p450_{scc}$ may be limiting to progesterone production

during luteinization. Concentrations of $p450_{scc}$ and its electron donor, adrenodoxin, were similar between large follicles and early (luteinizing) CL in the bovine, but these concentrations increased ~12 fold in stage II and III (mid to late luteal phase) corpora lutea, and this increase was not solely due to the increase in number of mitochondria (45). In primate granulosal cells obtained prior to the preovulatory gonadotropin surge, no $p450_{scc}$ activity was detected, even though mRNA for $p450_{scc}$ was present (46, 47). Possibly, the preovulatory gonadotropin surge provides the initial stimulus for $p450_{scc}$ expression, and once this signal is received, $p450_{scc}$ will be constitutively expressed throughout the luteal phase. In support of this theory, concentrations of $p450_{scc}$ mRNA increased rapidly following human chorionic gonadotropin (hCG)-induced luteinization of rat preovulatory follicles, and then $p450_{scc}$ expression was maintained through the remainder of the luteal phase (48). Collectively, although there is evidence that $p450_{scc}$ may limit progesterone synthesis during luteinization, it does not appear to be a limiting factor to steroidogenesis during the remainder of the luteal phase.

The expression of 3β-HSD remains constant during the majority of the luteal phase, but then decreases about the time of luteolysis in both sheep (49) and cows (50). Concentrations of 3β-HSD mRNA was similar in CL's collected at days 3, 6, 9, and 12 of the ovine luteal phase, but was lower in day 15 corpora lutea (49). In women, 3β-HSD is at the highest concentrations during the early luteal phase, but then decreases by the mid-luteal phase and remains low until luteolysis unless stimulated by hCG (42). In the preovulatory follicle, 3β-HSD is localized to the theca interna in both the sheep (11) and rat (51). Following ovulation, 3β-HSD activity was detected in large (granulosa-derived)

cells of the sheep (52) and rat (51). Thus, it appears that the preovulatory gonadotropin surge may stimulate expression of 3β-HSD in granulosal, and thus large luteal cells. Once luteinization is complete, 3β-HSD does not appear to be limiting to progesterone synthesis, with the possible exception of the late luteal phase. Incubating purified large and small ovine luteal cells with pregnenolone, and then measuring conversion to progesterone as a marker of 3β-HSD activity indicated that it is not limiting to progesterone synthesis (44, 53). It is accepted that in many species $p450_{scc}$ and 3β-HSD are not limiting steps in progesterone synthesis. Rather, it has been concluded that the rate-limiting step in progesterone production is the transport of cholesterol to the inner mitochondrial membrane where the $p450_{scc}$ complex resides (reviewed in 37, 54, 55)

Tropic Regulation of Luteal Function

Pituitary and/or hypothalamic hormones regulate growth and progesterone secretion of the CL in most species. Luteinizing hormone is the primary luteotropic hormone in most cases, although estradiol and prolactin are responsible for tropic regulation of CL function in some species. The requirement of LH for normal luteal function has been demonstrated by experiments involving hypophysectomy or specific removal of LH from the blood by treatment with antisera against LH (reviewed in 12). In ewes, hypophysectomy on day 5 of the estrous cycle prevented the increases in CL weight and serum concentrations of progesterone that normally occur during the luteal phase (56, 57). Luteinizing hormone replacement restored serum concentrations of progesterone, but weight of the CL remained lower than controls (56, 57). Luteinizing hormone and growth hormone (GH) replacement was required to maintain luteal weights and progesterone

secretion at levels observed in non-hypophysectomized ewes (57); thus GH also has a luteotropic effect in some species. Similar experiments involving treatment with antisera against LH have resulted in decreased luteal weight and/or luteal concentrations of progesterone in sheep (58), cattle (59), horses (60), and pregnant but not cycling swine (61). In the sow, the requirement for LH appears to be limited to pregnancy as hypophysectomy during the early luteal phase did not affect CL function (62). In primates, LH is also critical for CL function as demonstrated by treatment of monkeys with a GnRH antagonist, which caused significant reductions in serum concentrations of progesterone (43, 63).

Estradiol and prolactin have a role in CL function of some species. Prolactin is required for normal CL function in rodents and dogs (reviewed in 12). In dogs, reducing serum concentrations of prolactin by treatment with ergocryptine causes a significant reduction in progesterone secretion (64). Treatment of dogs with LH antisera also decreases serum concentrations of progesterone; thus, it appears that both LH and prolactin are required for normal progesterone synthesis in the dog (64). Estradiol is luteotropic in the rabbit and pig (reviewed in 8). In rabbits, estradiol is primarily responsible for normal CL function (65, 66). However, estradiol does not increase progesterone synthesis in rabbits, but the rabbit CL responds to LH with enhanced progesterone synthesis (67) similar to what is observed in many other species. In the pig, administration of estradiol causes CL growth and increased progesterone secretion (68). There may still be a requirement for LH in the pig as it is able to stimulate progesterone synthesis by luteal tissue *in vitro* (31). It is worth noting that although prolactin or estradiol are the primary regulators of CL

function in some species, LH also results in increased progesterone secretion in these species.

Luteolysis

Overview of Luteolysis

Luteolysis is physical destruction of the CL. This process is required for a female to ovulate again if pregnancy has not taken place, but luteolysis must be prevented if pregnancy has occurred so there will be a continued source of progesterone. The process culminating in luteolysis can be subdivided into two phases: 1.) a decrease in progesterone secretion, and 2.) structural demise of the CL (reviewed in 8, 69). It is worth noting that the decrease in progesterone secretion precedes lysis of the CL. Furthermore, structural demise of the CL can be prevented by restoring progesterone concentrations early in the luteolytic process of the bovine (70), ovine (71), rabbit (72), and primates (73, 74). Prostaglandin F2α (PGF2α) has been identified as the primary luteolytic hormone (reviews in 8, 69, and 75). The luteolytic properties of PGF2α were first demonstrated in pseudopregnant rats (76), and subsequently have been confirmed in many other species (reviewed in 12). Active or passive immunization to PGF2α prevents spontaneous luteolysis in the ewe (77-79), cow (79), and guinea pig (80). The source of PGF2α during luteolysis remains somewhat controversial. In domestic animal species, uterine PGF2α is required for luteolysis (reviewed in 12, 69, 75), but PGF2α from the CL may also be necessary (reviewed in 8). The uterus is not required for luteolysis in rhesus monkeys (81, 82), humans (83, 84), and dogs (85), but PGF2α infusion causes a transient decrease in progesterone secretion, the first indicator of ensuing luteolysis, by

the CL of these species (reviewed in 12). In women, although uterine PGF2α is not required for luteolysis (86), PGF2α is produced by the human CL (reviewed in 87) and may act in an autocrine/paracrine manner to cause luteolysis (reviewed in 88).

Decrease in Progesterone Synthesis

Prostaglandin F2α decreases progesterone secretion by the CL in ewes, cows, sows, primates, rats and rabbits (reviewed in 12). The effect of PGF2α to reduce serum concentrations of progesterone is most likely due to two factors; 1.) restricting luteal blood flow which would inhibit progesterone produced in the CL from entering the systemic circulation, and 2.) down-regulation of progesterone synthesis by luteal cells. There are several mechanisms whereby PGF2α may decrease progesterone synthesis in luteal cells: decreasing expression of receptors for luteotropic hormones, decreased activity or expression of the enzymes involved in progesterone biosynthesis, preventing uptake of cholesterol from extracellular sources, and inhibiting the transport of cholesterol to the $p450_{scc}$ complex (reviewed in 8). Prostaglandin F2α causes a decrease in the number of receptors for LH in rats (89), ewes (90), and cows (91), but these decreases in number of LH receptors occurred well after the drop in serum concentrations of progesterone. Thus, the effect of PGF2α on number of LH receptors appears to be a secondary effect rather than a primary cause of the inhibition of progesterone synthesis that occurs early in the luteolytic process. There is no effect of PGF2α on mRNA, protein, or activity of the $p450_{scc}$ enzyme complex (reviewed in 8). Prostaglandin F2α decreases mRNA for 3β-HSD in the sheep (49) and guinea pigs (92), but the concentrations of 3β-HSD protein and its enzyme activity were not affected for the first

24 hours following PGF2α treatment in the sheep (93) and cattle (94). Thus, similar to the effect of PGF2α on LH receptors, the decrease in 3β-HSD mRNA does not appear to contribute to the initial decrease in progesterone synthesis.

The role of PGF2α on lipoprotein uptake and utilization is controversial. The expression of LDL receptor mRNA decreases following PGF2α treatment in both the bovine (95) and ovine CL (96). However, mRNA for HDL binding protein increased in sheep following PGF2α treatment (96), and HDL is the preferred cholesterol substrate in ruminants (reviews in 8, 97). The activity of cholesterol esterase, an indicator of lipoprotein utilization, was not affected by PGF2α treatment in sheep (53). Furthermore, there was no effect of PGF2α on lipoprotein utilization in bovine luteal cells, and it was concluded that the inhibition of cholesterol conversion to progesterone occurs at the level of the mitochondria (98). This conclusion is interesting because, as stated previously, the rate-limiting step in progesterone synthesis is transport of cholesterol across the mitochondrial membrane to the $p450_{scc}$ enzyme complex. Thus, cholesterol transport across the mitochondrial membrane may be a primary point of regulation whereby PGF2α exerts its rapid negative effect on progesterone production.

Prostaglandin F2α binding to its receptor activates the protein kinase C (PKC) second messenger pathway (71, 99). Activation of PKC by PGF2α decreases progesterone synthesis, but PKC activity itself does not induce apoptosis (71). Prostaglandin F2α activation of PKC is specific for large luteal cells as PGF2α receptors are localized to this cell type (16), and PGF2α decreases progesterone synthesis by large luteal cells (99).

Ovine small luteal cells do not contain PGF2α receptors (16) or respond to PGF2α treatment with decreased progesterone secrection (53). However, pharmacological activation of PKC in small luteal cells inhibits protein kinase A (PKA)-induced progesterone production (53).

What hormone(s) may activate PKC in small luteal cells during luteolysis is not definitively known. One candidate is oxytocin, which has been shown to decrease progesterone synthesis by porcine (100, 101) and human (102) luteal tissue/cells, and oxytocin receptors have been identified in small luteal cells of the pig (100). Furthermore, PGF2α causes oxytocin release by the ovine CL during luteolysis (103), and ligand binding to the oxytocin receptor is believed to activate PKC (104). Prostaglandin F2α, or other hormonal activators of the PKC second messenger pathway, may decrease cholesterol transport to the $p450_{scc}$ enzyme complex by decreasing expression and/or causing post-translational modifications of cholesterol transport proteins, which will be discussed in more detail later.

Regardless of the mechanisms whereby PGF2α or other luteolytic hormones cause a decrease in progesterone synthesis, it is important to note that inhibition of progesterone secretion is the first symptom of oncoming luteolysis. Thus, declining serum concentrations of progesterone during luteolysis are not simply an effect of dying luteal cells, but may be necessary for luteolysis to ensue. A common concept in the literature is that inhibition of progesterone production results in "functional luteolysis", but this is a miss-use of terminology as luteolysis by definition is a "lysis or structural demise of the

corpus luteum" (8). This initial phase of the luteolytic process does not involve structural breakdown of the CL because following $PGF_{2\alpha}$ exposure in sheep, the number of luteal cells does not decrease (105), and morphologic indicators of apoptosis are not evident (106) until after serum concentrations of progesterone have diminished. Additionally, numerous studies have confirmed a time lag between the drop in progesterone secretion and apoptosis in other species (reviewed in 69). However, inhibition of progesterone secretion can not be excluded from the luteolytic process, as there is a growing body of evidence that this step is obligatory for the structural demise that follows.

Structural Demise of the Corpus Luteum

The physical lysis of the CL occurs by apoptosis, which is programmed cell death and differs from necrosis, which can result from physical trauma to a tissue (106). Prostaglandin F2α promotes apoptosis in luteal tissue (106). Morphologically, the first indicators that a cell is undergoing apoptosis include a decrease in cell size coupled with the appearance of apoptotic bodies, which are membrane-enclosed vesicles that may contain nuclear fragments and cytosolic fractions (107). Immune cells will phagocytose apoptotic bodies in luteal tissue (108). Other morphologic indices of apoptosis include condensation of chromatin, cytoplasmic organelle compaction, membrane blebbing, and appearance of pyknotic nuclei due to contraction of the nucleus (reviewed in 69). Additionally, during apoptosis DNA fragmentation occurs due to the activation of Ca^{2+}-dependent endonucleases (109). This causes the formation of characteristic 185-bp fragments, or multiples of 185-bp, known as oligonucleosomes (110), which are easily visualized by agarose gel electrophoresis. Oligonucleosome formation during apoptosis

differs from nonspecific DNA fragmentation that occurs as a result of necrosis (69). Prostaglandin F2α stimulates oligonuclosome formation in luteal cells from many species including cattle, sheep, humans, rats, and pigs (reviewed in 8).

Prostaglandin F2α has cytotoxic effects on purified large, but not small, ovine luteal cells (111). This is not surprising as PGF2α receptors are localized to large cells (16). The number and size of large luteal cells decreases within 24 hours of PGF2α administration in ewes, and precedes a decrease in number of small luteal cells (105). Prostaglandin F2α increases concentrations of intracellular free calcium (112), which may mediate the cytotoxic effects via apoptotic mechanisms (106). This increase in free calcium within the cell has been demonstrated in human, bovine, and ovine luteal cells (reviewed in 69). While increases in intracellular concentrations of free calcium may be the primary initiator of apoptosis in large cells, activation of PKC induces the expression of pro-apoptotic proteins in other cell types (reviewed in 113), and therefore may play a role in initiating apoptosis in both large and small cells.

In ewes, a rapid decrease in luteal blood flow occurs following PGF2α administration (114). This decrease in blood flow may contribute to structural breakdown by restricting available nutrients and luteotropic support (115). This is probably caused by the effect of PGF2α on endothelial cells as this cell type contains receptors for PGF2α (116), and PGF2α causes apoptosis of endothelial cells (106, 117). However, a decrease in blood flow in response to PGF2α does not appear to occur in all species as ovarian blood flow is not affected following a luteolytic dose of PGF2α in rats (118).

Role of the Immune System in Luteolysis

The immune system appears to play an active role in lysis of the CL. An immunosuppressive dose of dexamethasone delays natural, but not PGF2α-induced luteolysis in rats (119). Additionally, splenectomy of rats causes elevated serum concentrations of progesterone during the time of normal luteal regression, an effect that was reversible with injection of splenocytes (120). Lymphocytes are found within the bovine CL at approximately day 14 of the estrous cycle, 3-4 days prior to luteolysis (121). In ewes, eosinophils migrate to luteal tissue that has been exposed to PGF2α more readily than in control ewes (122). Many studies have found an increase in number of lymphocytes and macrophages in luteal tissue during the time of luteolysis (reviewed in 123). Macrophages are necessary for phagocytosis of dead or dying luteal cells, which will protect surrounding ovarian tissue from inflammation (123). However, they also appear to play a role in causing luteolysis via cytokine-mediated inhibition of steroidogenesis, and stimulation of PGF2α secretion by the CL (8). Recruitment of macrophages to the CL is mediated by monocyte chemoattractant protein-1 (MCP-1). The expression of MCP-1 increases during the time of luteolysis in rats (124, 125), rabbits (126), and cows (127), and mRNA for MCP-1 increases in ewes after PGF2α injection (128, 129). Macrophages and other immune cells will secrete cytokines including interferon-γ (IFN-γ), interleukin-1β (IL-1β), and tumour necrosis factor-α (TNF-α), which together can inhibit progesterone synthesis and increase luteal PGF2α secretion (reviewed in 8, 123). The inhibition of progesterone secretion is not dependent

on PGF2α release as progesterone synthesis still decreases in the presence of indomethacin, a prostaglandin synthesis inhibitor (130).

Major histocompatibility complex (MHC) genes encode cell surface glycoproteins. The MHC glycoproteins "present" peptides on the plasma membrane, and T lymphocytes determine if the cell is self or non-self depending on whether or not the peptide is foreign (123). There are two types of MHC glycoproteins designated class I and class II (75). Class I MHC molecules are expressed on nearly all nucleated cells (75). Class I MHC molecules present peptides that are recognized by CD8+ T lymphocytes, and these lymphocytes will have cytotoxic activity toward the antigen-presenting cell if the presented peptide is recognized as non-self (123). Class II molecules are generally expressed by immune cells such as macrophages and B cells, but there are several examples of somatic cell expression of class II molecules (reviewed in 123). This is a common feature of most autoimmune diseases (131). Cytokines can induce expression of both classes of MHC glycoproteins on luteal cells (123). Exposure to TNF-α or IFN-γ increases the expression of class I MHC molecules (132), and IFN-γ also induces class II MHC expression (133). Class I MHC molecules are present on luteal cells although their expression does not change during the luteal phase, but class II MHC expression increases in the CL at approximately the time of luteolysis (134). Additionally, expression of class II molecules is significantly increased by PGF2α (134), and MHC expression (class I and class II) is significantly lower in corpora lutea from pregnant compared to non-pregnant animals in both cattle (134) and sheep (135). Luteal cells stimulate T cell proliferation, indicating that MHC molecules on luteal cells are active

immunologically (136). In summary, there is compelling evidence that the immune system is actively involved in luteolysis, an involvement beyond merely preventing a potentially inflammatory response induced by dying luteal tissue, and that luteal cells themselves may initiate a transient autoimmune response during luteolysis.

Maternal Recognition of Pregnancy

Rescue of the Corpus Luteum

Progesterone is required for maintenance of pregnancy in all-mammalian species (7). During pregnancy, the CL and/or placenta produce progesterone. In many species, luteal progesterone is no longer required once the placenta has developed and is producing sufficient quantities of progesterone to support the pregnancy, a process known as the luteal/placental shift (7). The period of time when this shift occurs varies greatly between species (8). However, during early pregnancy when the placenta has not yet acquired the steroidogenic capacity to maintain pregnancy, luteal progesterone is essential to survival of the embryo, and in some species a functional CL is required for the length of gestation (88). In most mammals, the gestation period exceeds the length of a normal luteal phase. This means that if pregnancy has occurred, a mechanism whereby the lifespan of the CL is extended must ensure that progesterone will continue to be produced. This process, in which the conceptus provides a biochemical signal to the dam notifying her of its presence, is known as maternal recognition of pregnancy (137). Successful maternal recognition of pregnancy involves preventing luteolysis, a process often referred to as "rescue of the CL" (7, 137). The mechanisms causing CL rescue differ between species, although in general they involve a specific biochemical signal sent during a critical

period of time (8). Regardless of species, the signal produced by the conceptus can rescue the CL via two mechanisms: 1.) preventing the secretion or luteolytic actions of PGF2α, and 2.) providing a luteotropic signal (8). These two mechanisms are not mutually exclusive, as the signal for pregnancy in some species will utilize both.

Species Differences in Maternal Recognition of Pregnancy

Chorionic gonadotropin (CG) is the hormone produced by the developing conceptus that causes maternal recognition of pregnancy in women and non-human primates. In women, the embryo implants in the uterus approximately 6-7 days post-ovulation (7). Following implantation, the trophoblastic cells of the conceptus begin to secrete CG into the maternal blood (138). Chorionic gonadotropin is structurally similar to LH and binds to LH receptors, thus stimulating progesterone synthesis by the CL (139). The primary difference between CG and LH is that CG has a longer half-life due to more extensive glycosylation (8). Due to its longer half-life, CG is more biologically active than LH as evidenced by the finding that in sheep hCG occupies the LH receptor longer than LH (140). Therefore, rescue of the CL in primates involves a luteotrophic effect.

Interferon-tau (IFN-τ) is the signal for maintenance of luteal function during pregnancy in ruminants (137). Interferon-tau is produced by the trophoblastic cells of the conceptus, and must be present between days 12-15 post-estrus in sheep (141, 142) and days 14-17 in cattle (143), encompassing the period of normal luteolysis in each respective species. In ruminants, IFN-τ rescues the CL by preventing pulsatile secretion of PGF2α in sheep (144) and cattle (145-147). Interferon-tau inhibits PGF2α production by decreasing the

number of oxytocin receptors in the endometrium (148). The decrease in number of oxytocin receptors occurs due to three factors: 1.) IFN-τ inhibits transcription of the genes for estradiol and oxytocin receptors, 2.) the reduction in number of estradiol receptors suppresses estradiol-induced expression of oxytocin receptors, and 3.) progesterone, which is still produced because of CL rescue, further down-regulates expression of the genes encoding receptors for estradiol and oxytocin (reviewed in 8). Ultimately, the reduced number of oxytocin receptors in the endometrium suppresses oxytocin-mediated PGF2α release from the uterus (8). Interferon-tau also causes a shift in prostaglandin synthesis to favor production of PGE2 over PGF2α (149). Prostaglandin E2 amplifies the increase in progesterone synthesis induced by HDL supplementation in bovine luteal cells (150). Additionally, there is a positive feedback loop in the cow whereby PGE2 stimulates progesterone production and vice versa (150).

Although luteolysis in the pig occurs by a similar mechanism as in ruminants, which relies on uterine PGF2α release, the mechanisms that cause maternal recognition of pregnancy in swine are very different (151). The biochemical signal produced by porcine conceptuses that establishes pregnancy is estradiol-17β, which is produced by approximately day 11 of pregnancy but must be present by day 12 (151). The mechanisms causing maternal recognition of pregnancy in the pig are not well understood, and the effects of estradiol during maternal recognition of pregnancy are complex. It has been theorized that estradiol causes PGF2α to be released into the uterine lumen rather than the blood (152). This would prevent PGF2α delivery to the CL. This theory is backed by the finding that concentrations of PGF2α are higher in the uterine

lumen of pregnant versus cycling swine (152). The mechanism whereby estradiol may change the secretion of PGF2α is not well understood. Another effect of estradiol that protects the CL is inducing production of PGE2. Prostaglandin E2 has a luteotrophic/anti-luteolytic effect in swine (153, 154). Prostaglandin E2 increases, while PGF2α decreases, progesterone production in porcine luteal cells (155). Treatment of porcine luteal cells collected from regressing corpora lutea with increasing ratios of PGE2:PGF2α prevents the negative effect of PGF2α on progesterone secretion (155). Furthermore, estradiol increases the ratio of PGE2:PGF2α released from the endometrium in swine (151).

Horses produce CG, however not until approximately day 35 of pregnancy. Thus, CG does not play a role in maternal recognition of pregnancy as this is past the period when luteolysis occurs in non-conception cycles (8). It appears that the conceptus, possibly via its movement within the uterus, alters the ratio of prostaglandin E2 (PGE2) versus PGF2α (8) produced. Prostaglandin E2 is luteotropic, and maintains the CL until CG is produced. The role of CG in horses is different than in primates. In horses, CG promotes follicular growth and ovulation, thus creating many accessory corpora lutea (up to 70 in a single mare) (8). These accessory CL's will provide progesterone until approximately day 160 of pregnancy when the placenta takes over as the primary source of progesterone (8). Thus, in the mare, CG can stimulate progesterone synthesis not only via binding to the LH receptor, but increases serum concentrations of progesterone by causing formation of accessory CL's. Therefore, CG in the mare will maintain pregnancy by increasing progesterone production similar to the effect of CG in primates, but it is not the factor causing maternal recognition of pregnancy.

In rodents, stimulation of the cervix by mating or artificial excitation initiates a neuroendocrine reflex arc that causes release of prolactin, LH, and FSH (156). Prolactin from the pituitary maintains the CL for 10-12 days (157). Prolactin is produced in a diurnal pattern for the first 8 days following cervical stimulation, plus a nocturnal release for an additional 2 days; if no embryos are present, a new estrous cycle begins on day 12 (157). In pregnant rats, placental lactogen produced by the conceptus is detectable on day 8, corresponding to the end of the diurnal release of prolactin, and is maximal by day 10 (158). Placental lactogen binds to the prolactin receptor, which increases progesterone production and will maintain the CL for the remainder of gestation (159). Additionally, there is an increase in androgen production, which is subsequently converted to estradiol by the placenta (8). The CL contains receptors for estradiol, and ligand binding causes an increase in progesterone synthesis and cell hypertrophy (160).

In species in which maternal recognition of pregnancy involves blocking the synthesis or activity of PGF2α such as ruminants and swine, there is considerable evidence that during maternal recognition of pregnancy, these species undergo a luteotrophic effect as well. The luteotrophic effect in non-primate species most often occurs due to PGE2. Coupled with data indicating that a decrease in progesterone synthesis by the CL precedes structural lysis, and that an inhibition of progesterone synthesis is pre-requisite for structural demise of the CL, it is possible that a stimulation of progesterone secretion is critical to successful maternal recognition of pregnancy in nearly all species. Thus, progesterone itself may not only be necessary to maintain the pregnancy, but may also

play a crucial role in maintaining its own synthesis by aiding in CL rescue during early pregnancy.

One question regarding maternal recognition of pregnancy is why primates exclusively utilize a luteotropic signal to rescue the CL, while other species like ruminants use both luteotrophic and anti-luteolytic mechanisms to prevent luteolysis in early pregnancy? The answer may be explained by timing of embryo attachment to the uterus relative to luteolysis. In humans, implantation occurs approximately 6-7 days after ovulation (7), and with a 14-day average luteal phase, implantation takes place approximately a week before luteolysis occurs. Thus, it makes sense that in humans the signal for maternal recognition of pregnancy does not work through anti-luteolytic mechanisms. As appears to be the case in humans, it is possible that a luteotropic signal itself is sufficient to establish pregnancy if released well before the beginning of the luteolytic cascade. This notion is backed by the finding that if implantation is delayed in women, the rates of embryonic loss increase (161). In other species in which a luteotropic signal is not exclusively used to establish pregnancy, embryo attachment to the uterus occurs during or after the normal period of luteolysis. For example, cows have an 18-day average luteal phase, with implantation occurring approximately 18-22 days after ovulation (7). Therefore, implantation in the bovine does not happen until luteolysis would have already occurred in non-conception cycles. If implantation has not taken place prior to the beginning of luteolysis, the embryo does not have direct access to the maternal blood supply and thus does not have an anatomical pathway whereby it can send a luteotropic signal to the CL. Also, the uterus is required for luteolysis in cattle (12), so it is logical

that the signal for maternal recognition of pregnancy in cattle exerts its effects directly in the uterus. A luteotrophic effect may be required to maintain CL function in early pregnancy in cattle, but if implantation has not occurred, this effect must occur by indirect mechanisms. The biochemical signal for pregnancy in cattle, IFN-τ, provides a luteotrophic effect indirectly by increasing PGE2 production by the uterus (149). In summary, although there are considerable differences between species in the mechanisms that prevent luteolysis and establish pregnancy, a luteotrophic effect may be required in all species, and may be able to rescue the CL if it occurs with sufficient time prior to the initiation of luteolysis.

Effects of Progesterone on Rescue of the Corpus Luteum

Progesterone Receptors in the Corpus Luteum

It has been proposed that progesterone is a 'universal luteotrophin' that promotes its own synthesis in many species (162). However, studies to elucidate the mechanisms whereby progesterone regulates CL function are difficult to perform because of the high concentrations of progesterone in luteal tissue. This hinders the use of methods normally used to study the mechanisms of steroid action in non-steroidogenic tissues (163). Nonetheless, studies since the late 1980's have shed some light on the role of progesterone in CL regulation. The nuclear progesterone receptor (PR) has been identified and is co-localized to steroidogenic cells (as determined by 3β-HSD histochemical stain) in the primate CL (164). Furthermore, PR expression is stimulated in granulosal cells of the preovulatory follicle following the preovulatory gonadotropin surge in rodents, domestic animals and primates (reviewed in 163). In species with long

luteal phases such as domestic animals and primates, PR is expressed in the CL during the luteal phase and early pregnancy, but PR appears to be transiently expressed only in the preovulatory follicle of the rodent as PR protein has not been identified in the rat CL (reviewed in 163). Identification of PR during the luteal phase indicates that progesterone may be able to regulate CL function in an autocrine/paracrine manner via classic genomic mechanisms. There is also mounting evidence for membrane progesterone receptors that can act via genomic or non-genomic mechanisms to alter cell function (163). Membrane receptors for progesterone have been identified in the bovine CL (165), and a progestin membrane receptor regulates oocyte meiotic maturation in fish (166). So far though, the role of membrane progesterone receptors in CL function is not well understood, and the investigation into non-genomic effects of steroids via membrane receptors is currently an area of intense research.

Interactions with Other Hormone Receptors

Due to the high concentrations of progesterone within luteal tissue, it is possible that progesterone can have effects via low-affinity binding to other steroid receptors. Progesterone binding to the glucocorticoid receptor in rodents has been reported, which caused a decrease in 20α-hydroxysteroid dehydrogenase protein, an enzyme that metabolizes progesterone during luteolysis in the rat (167). Progesterone may also cause biological effects through peptide hormone receptors. Progesterone acts as an oxytocin receptor antagonist by competing with oxytocin for binding to the oxytocin receptor (168, 169). This finding is especially interesting regarding the possible role of oxytocin in apoptosis of small steroidogenic luteal cells.

Progesterone Depletion/Replacement Models

Steroid depletion/replacement methods have been employed to study the effects of progesterone on CL function. Hypophysectomy or treatment with gonadotropin-releasing hormone (GnRH) antagonists block LH secretion and consequently inhibit progesterone production by the CL. Progesterone synthesis is also prevented by treatment with the 3β-HSD inhibitor trilostane or the $p450_{scc}$ inhibitor aminoglutethimide. Progestins can be added back into the experimental system to study their effects (163). When trilostane is administered to monkeys during the mid-luteal phase, serum concentrations of progesterone rapidly decline as expected, but progesterone concentrations remained low after removal of the 3β-HSD inhibitor and these monkeys displayed a premature onset of menses (170). This effect on progesterone synthesis differed from the effect of trilostane on other steroids as cortisol concentrations decreased during the treatment period as well, but they returned to normal and adrenal gland function appeared unaffected following cessation of treatment (170). Corpora lutea collected from trilostane treated monkeys displayed morphologic indices of apoptosis such as pyknotic nuclei and more abundant immune cells compared to control tissue (170). These data provide evidence that progesterone is needed to maintain the structure and/or function of the CL during the luteal phase. To address the possibility that trilostane indirectly compromised CL structure/function by changing steroid feedback on pituitary LH secretion, a 'gonadotropin-clamped' model was used which involved GnRH antagonists and LH replacement (171). This allowed the effects of gonadotropin withdrawal to be

distinguished from the effects of steroid depletion, and this model confirmed earlier conclusions that progesterone is required to maintain the CL during the luteal phase.

Artificial Pregnancy Models

The role of progesterone in CL rescue has been tested using artificial pregnancy models in primates (172, 173). Administration of hCG to monkeys maintained progesterone secretion and extended the lifespan of the CL as expected (173). However, co-treatment with trilostane caused a decrease in luteal mass, increased formation of pyknotic nuclei, and a greater number of lymphoid cells similar to time-matched controls that were undergoing luteolysis (173). Thus, it was concluded that progesterone itself mediates at least some of the effects of hCG to rescue the CL. Although studies investigating the role of progesterone on CL rescue are limited and have been performed primarily in primates, a possible role for progesterone in CL rescue of the bovine has been reported (174). In this study, bovine luteal cells isolated from CL's of early pregnancy were treated with aminoglutethimide, which prevented progesterone synthesis. The decrease in progesterone concentrations promoted apoptosis as evidenced by oligonucleosome formation and results of Hoeschst 33258 staining, which distinguishes cells undergoing apoptosis on the basis of nuclear condensation, morphology, and increased fluorescence (174). Interestingly, progesterone replacement prevented appearance of apoptotic indicators. Furthermore, the progesterone receptor antagonists, RU486 and onapristone, caused an increase in oligonucleosome formation without affecting media concentrations of progesterone (174), and it was concluded that progesterone inhibits apoptosis of luteal cells via a receptor-dependent mechanism in cows. These data in cattle are intriguing

because a luteotropic signal is not the primary mechanism causing CL rescue in this species, so a role for progesterone in CL rescue is somewhat surprising. Thus, there is both *in vivo* and *in vitro* evidence for a role of progesterone in CL maintenance and rescue.

Progesterone and the Immune System

Progesterone can modulate immune system function. It has been hypothesized that rescue of the CL during early pregnancy must involve a local immunosuppressive mechanism (175). Indeed, lymphocytes and macrophages are recruited into the CL during non-conception cycles, but the recruitment of these immune cells does not occur in CL's of the same age if pregnancy has occurred (121, 176). Also, expression of MHC glycoproteins is lower in CL's of pregnant versus non-pregnant animals (134, 135). Thus, it appears that immunosuppressive mechanism(s) are acting to rescue the CL during early pregnancy. Progesterone is immunosuppressive and is known to inhibit lymphocyte proliferation and function (reviews in 177-179). Additionally, progesterone can block cytokine action in the CL. Cytokine-induced PGF2α synthesis is prevented by progesterone, and progesterone will block IFN-γ-induced expression of MHC molecules on luteal cells (180). Therefore, maintaining high concentrations of progesterone within the CL may cause immunosuppression that contributes to CL rescue during early pregnancy. If luteal concentrations of progesterone decrease, as normally occurs just prior to luteolysis, the inhibitory effect of progesterone on immune system function would be removed and the CL will regress, at least in part, due to immune attack (123).

Early Embryonic Wastage

A high percentage of early embryonic mortality occurs in mammals before placentation is complete (137), and consequently before the placenta is producing enough progesterone to maintain the pregnancy. Therefore, embryonic losses during this period could be due to insufficient progesterone production by the CL, possibly caused by luteal insufficiency or failure to rescue the CL (181, 182). In cattle, 20-50% of all embryos are lost during pregnancy (182, 183). Also in ruminants, the majority of embryonic losses occur in the first 3 weeks post-fertilization (184, 185), which incidentally is the approximate length of the luteal phase in non-conception cycles. In pigs, 30-40% of conceptuses are lost during pregnancy with the majority of losses occurring before placentation is complete (186). The percentage of embryonic wastage may be even higher in humans. It has been estimated that up to two-thirds of embryos are lost in healthy women attempting natural conception (187), and an even higher percentage of loss occurs in women using assisted reproduction (188). The majority of these losses in women occurs within the period of a normal luteal phase and therefore usually goes unnoticed, once again indicating a failure to rescue the CL as a possible cause.

Baird *et al.* (2003) [161] studied 120 naturally conceived human pregnancies in women that had no known fertility problems. Daily urine samples were analyzed for hCG (to determine time of implantation) and the major urinary progesterone metabolite, pregnanediol-3-glucuronide (PdG). Of these women, 77 developed clinical pregnancies (defined as lasting at least 6 weeks from last menstrual period) while 43 lost the pregnancy within this time frame. In this study, 6 weeks was selected as the cut-off point

for definition of pregnancy as this is the approximate time of the luteal/placenta shift in women (161), so any loss of pregnancy prior to 6 weeks could possibly be credited to inadequate luteal function. The losses were attributed primarily to insufficient hCG release, or low serum concentrations of progesterone as indicated by urinary levels of PdG. It was discovered that on average, women who developed clinical pregnancy had significantly higher concentrations of PdG than non-conception cycles at 10 days post-ovulation. Additionally, they observed that the majority of these women experienced a significant rise in PdG one day after implantation that continued to rise gradually until a week after first detection of urinary hCG. On the other hand, the 43 women with early losses had lower PdG levels after the first week following implantation than those in the clinical pregnancy group, although their concentrations of PdG were still higher than non-conception cycles. Thus, although progesterone synthesis had been stimulated, it may not have been sufficient to maintain the pregnancy in some cases. The risk of an early loss of pregnancy was calculated to be 5% in women who exhibited a marked increase in progesterone synthesis at implantation, with the risk increasing to 25% if progesterone secretion was simply maintained, and nearly 100% if serum concentrations of progesterone declined. This indicates that inadequate progesterone secretion, which may be due to either insufficient hCG secretion from the embryo or an insufficient response by the corpus luteum to hCG, is highly related to early pregnancy losses in women.

Certainly, factors other than inadequate progesterone production may contribute to the high amount of embryonic wastage that occurs in mammals, although this appears to be

one of the leading causes. It is important to note that simply maintaining progesterone production during early pregnancy may not be sufficient to rescue the CL, as illustrated in the Baird *et al.* (2003) [161] study in which women who lost their pregnancy still had higher concentrations of PdG compared to non-conception cycles, but lower levels of PdG than their counterparts who established a clinical pregnancy. Thus, an increase, not just maintenance, of progesterone secretion may be required to prevent luteolysis in early pregnancy. Because this study was performed in women, the question arises that if progesterone synthesis is stimulated following implantation of the conceptus, is this simply a side-effect of hCG and progesterone itself does not play a role in CL rescue? However, it seems unlikely that progesterone itself will not contribute to CL rescue as there have been several studies in different species that indicate it plays a crucial role in preventing luteolysis, and lysis of the CL occurs after serum concentrations of progesterone have decreased. Whether an increase in serum concentrations of progesterone following implantation is required in species that do not use a luteotropic signal to establish pregnancy is more debatable. However, many of these species still experience a luteotrophic effect during CL rescue, and thus progesterone itself may play a crucial role in this process.

Progesterone Synthesis in Small and Large Steroidogenic Luteal Cells

Progesterone synthesis, and apparently its regulation, occurs very differently in the two-steroidogenic cell types of the CL. Large and small steroidogenic cells have been identified in corpora lutea of virtually all mammals studied (10), and their pattern of progesterone secretion is strikingly similar regardless of species. The rate-limiting step in

steroidogenesis is transport of cholesterol from the outer to inner mitochondrial membrane where the $p450_{scc}$ enzyme complex resides, and this step is also the most acutely regulated by second messengers. Protein kinase-A stimulates while PKC inhibits progesterone synthesis in the CL (14). The most notable hormones that activate the PKA and PKC pathways in the CL are LH and PGF2α respectively (8), which are also the primary luteotropic and luteolytic hormones in most species. These two hormones, along with the kinase pathways they ultimately activate, have different effects on progesterone synthesis in the two steroidogenic cell types.

Small steroidogenic luteal cells have lower basal production of progesterone compared to large cells. However, they can increase their progesterone secretion 5-20 fold with LH/hCG stimulation, and this effect can be mimicked with pharmacological activators of the PKA pathway such as dibutryl cAMP and forskolin (16). Large cells produce high concentrations of progesterone constitutively (~20 fold higher than an equal number of small cells in sheep) (16), but do not respond to LH/hCG, or non-hormonal activators of PKA, with increased progesterone synthesis. In small cells, ligand binding to the LH receptor causes an increase in intracellular concentrations of cAMP, which in turn activate PKA by binding to the regulatory subunit and causing the catalytic subunit to dissociate (14). Protein kinase-A increases progesterone synthesis by 3 mechanisms: 1.) increasing free cholesterol within the cell via activation of cholesterol esterase (hormone-sensitive lipase), 2.) increasing lipoprotein uptake, and 3.) stimulating increased cholesterol transport across the mitochondrial membrane to the $p450_{scc}$ enzyme complex by a mechanism that is not well understood (reviewed in 14). Protein kinase-C inhibits

progesterone synthesis in both cell types. Activation of PKC with phorbol esters decreases basal progesterone synthesis by large cells, and prevents LH-stimulated progesterone production in small cells (53). Prostaglandin F2α activates PKC in large but not small steroidogenic cells as is expected because PGF2α receptors are present in large and not small cells (14, 16). The biological activator of PKC in small cells is not known.

An interesting "dualism" exists between the two steroidogenic cell types in the CL. Large cells appear to be the "pace-setters" of luteal progesterone synthesis. They produce large quantities of progesterone constitutively, and their primary regulation is negative via PGF2α and PKC. Small cells have lower basal progesterone synthesis compared to large cells, and their primary regulation is stimulatory through the LH receptor and PKA. Progesterone synthesis in large cells can not be up-regulated, with the possible exception of stimulation by PGE2. Reports of PGE2 effects on progesterone production in luteal cells are conflicting. In gilts, PGE2 stimulates progesterone synthesis by both large and small steroidogenic cells (189). In sheep, PGE2 stimulates progesterone production by large and not small luteal cells (190), but in another study the results were opposite as PGE2 stimulated progesterone synthesis in small but not large luteal cells (191). This discrepancy in sheep may have been because different methods were employed to separate the large and small cells in the two studies, and therefore the purity of the cell populations studied may have been different. It would be interesting to know the exact role of PGE2 on progesterone production by both steroidogenic cell types due to its possible role in maternal recognition of pregnancy in some species. However, one common result in those studies is that the stimulatory effect of PGE2 does not involve the

cAMP/PKA pathway (189, 190, reviewed in 8), so the mechanism is most likely different than the better understood LH receptor pathway. This is significant because LH is the primary luteotropic hormone; ligand binding to the LH receptor activates PKA, and PKA increases the rate-limiting step in steroidogenesis, transport of cholesterol across the mitochondrial membrane. It appears that only small cells have the ability to respond to PKA with enhanced steroidogenesis. Thus, the majority of progesterone produced during the luteal phase originates from large cells (estimated at ~80% from large cells in sheep) (192) due to their higher basal secretion of progesterone. If pregnancy does not occur, serum concentrations of progesterone will decrease, primarily due to PGF2α binding to its receptor on large cells, and luteolysis will ensue. Small cells contribute relatively little to circulating concentrations of progesterone during the luteal phase, but have the ability to increase their progesterone synthesis dramatically. This contribution by small cells may be especially important during CL rescue if pregnancy occurs. Regardless of species differences in the mechanism that causes maternal recognition of pregnancy, it appears that progesterone itself is required for CL rescue, and large cells are already at or near their peak progesterone secreting ability, so the small cells can meet any requirement for additional progesterone. Thus, it is important to understand the mechanism(s) behind constitutive progesterone production in large cells, which in turn may lead to techniques that stimulate progesterone synthesis by small cells during CL rescue. Given that the rate-limiting step in steroidogenesis is transport of cholesterol across the mitochondrial membrane, and the cAMP/PKA pathway is known to stimulate this transport, this is one logical area to investigate differences in steroidogenesis between small and large cells.

Cholesterol Transport across the Mitochondrial Membrane

Identification of Steroidogenic Acute Regulatory Protein

Due to the hydrophobic properties of cholesterol, it is logical that cholesterol can not simply diffuse through the cytoplasm, enter the hydrophobic outer mitochondrial membrane, diffuse out of the membrane interior where it is most soluble into the aqueous intermembrane space, and then through the inner mitochondrial membrane until it reaches the $p450_{scc}$ enzyme system. This kind of simple diffusion would be too slow to provide adequate substrate for cells producing large quantities of steroids. Rather, a system of transport proteins must be in place for cholesterol to be delivered rapidly to $p450_{scc}$.

The acute regulation of cholesterol transport to the inner mitochondrial membrane was first studied in adrenal tissue where it was discovered that ACTH-induced stimulation of corticoid synthesis was sensitive to cycloheximide, and thus dependant on de novo protein synthesis (reviewed in 193). It was discovered that the ACTH-regulated step was downstream of cholesterol ester hydrolysis but proximal to the cleavage of cholesterol by $p450_{scc}$ enzyme (194). Further studies provided evidence that the cycloheximide-sensitive step was located at the level of the mitochondria (195), and inhibition of protein synthesis had no effect on the activity of $p450_{scc}$ (196). Therefore, it was concluded that cycloheximide inhibited steroidogenesis by preventing expression of a protein that was responsible for transfer of cholesterol from the outer to inner mitochondrial membrane. Evidently, the protein responsible for acute stimulation of steroidogenesis is short lived,

so criteria for identification were that it is cycloheximide sensitive and highly labile (reviewed in 193).

Several proteins have been investigated as potential candidates for the cycloheximide sensitive factor responsible for acute regulation of steroidogenesis. The most widely accepted protein for this role is steroidogenic acute regulatory protein (StAR). Evidence that StAR is the acute regulatory factor first came with the discovery of 30 KDa phosphoproteins in rat and mouse adrenocortical cells that appeared following ACTH-stimulation, and these phosphoproteins may have consisted of different forms of the same protein (197-200). Phosphoproteins of approximately the same size were discovered in luteal cells, and their abundance increased following LH or cAMP treatment and increased steroidogenesis (201, 202). These phosphoproteins were also described in mouse and rat leydig cells (203-208). Together, the studies in the different steroidogenic cell types indicated that appearance of the 30 KDa phosphoproteins was highly correlated with hormonal stimulation and increased steroidogenesis, the proteins were localized to the mitochondria and consisted of several forms of a 30 KDa protein, and that synthesis of these proteins was sensitive to cycloheximide. These 30 KDa phosphoproteins were processed from a 37 KDa precursor, which contained an N-terminal mitochondrial targeting sequence (198, 204). The half-life of the 37 KDa precursor was calculated to be in the minute range (198), thus meeting the criterion that the factor responsible for acute regulation of steroidogenesis is highly labile. The cDNA for the 30 KDa protein was cloned from mouse leydig tumor cells (MA-10) in 1994 (209). Sequence analysis provided evidence that the cloned cDNA encoded a novel protein, and transient

transfection of MA-10 cells with this cDNA resulted in a significant increase in steroid production without hormonal stimulation (209). This was the first direct evidence linking the mitochondrial phosphoproteins with steroidogenesis. Thus, the protein was named StAR (209).

Congenital lipoid adrenal hyperplasia (lipoid CAH), a potentially lethal condition that causes severely reduced adrenal and gonadal steroid synthesis, is due to genomic mutations in the StAR gene (210). Lipoid CAH was originally believed to be due to mutations in the $p450_{scc}$ enzyme (211), but it was later determined that $p450_{scc}$ was normal in patients with the disease (212). Expression of the StAR cDNA cloned from patients with lipoid CAH in Cos-1 cells demonstrated that the mutated StAR protein was unable to promote normal steroidogenesis (210). While steroidogenesis was absent in cells containing the mutant protein, addition of 20α-hydroxycholesterol which can cross the mitochondrial membrane without the aid of transport proteins, returned pregnenolone levels to normal indicating that the transfected $p450_{scc}$ enzyme complex was functioning normally (210). Targeted disruption of the StAR gene was used to produce a knockout line of mice for investigating the *in vivo* role of StAR (213). All knockout mice displayed female external genitalia regardless of the genotype, the same as occurs in humans with lipoid CAH. Furthermore, death occurred within a short period of time after birth due to adrenal insufficiency. If the animals were rescued by mineralocorticoid therapy, the adrenals and testes contained large lipid deposits indicating an inability to convert cholesterol to pregnenolone, even though $p450_{scc}$ activity remained normal.

Mechanism of StAR Action

The exact mechanism whereby StAR mediates cholesterol transport across the mitochondrial membrane is still unknown. Because StAR is found in large quantities at the inner mitochondrial membrane, and due to the presence of a mitochondrial targeting sequence in the 37 KDa form, one theory is the mitochondrial targeting sequence targets StAR to the outer mitochondrial membrane and initiates import and processing (reviewed in 193). Possibly, contact sites between the outer and inner mitochondrial membranes would mediate or allow StAR transport and thus cholesterol as well. In this model, the 37 KDa form is active and processing to the 30 KDa form would inactivate StAR. However, several subsequent studies have demonstrated that deletion of the N-terminal mitochondrial targeting sequence has no effect on steroidogenesis, even though mutated StAR became localized to the outer and not inner mitochondrial membrane (214-216). Thus, import of StAR protein may not be required for cholesterol transfer to $p450_{scc}$, and there are possible roles for other proteins in cholesterol transport. The importance of the mitochondrial targeting sequence is unknown, and which form of StAR is active, the 37 or 30 KDa forms or both, remains debatable. While the N-terminal region of StAR does not appear to be required for cholesterol transport, truncation of the C-terminus causes a dramatic inhibition of StAR activity. Removing 28 amino acids from the C-terminus completely inhibits steroidogenesis (215), while removal of up to 62 amino acids from the N-terminus has no effect on steroid production. Not surprisingly, all known mutations that cause lipoid CAH occur in the final 40% of the protein nearest the C-terminus (217).

Peripheral-Type Benzodiazepine Receptors and Endozepine

Another protein system that may assist in transport of cholesterol from the outer to inner mitochondrial membrane is the peripheral-type benzodiazepine receptor (PBR)/endozepine system. The PBR is localized to the outer mitochondrial membrane (218, 219), and ligands for PBR stimulate steroidogenesis (218, 220). Targeted disruption of the PBR gene in R2C leydig cells blocked steroidogenesis, while re-introduction of the receptor returned steroid production to normal (221). Additionally, targeted deletion of the PBR gene *in vivo* is embryonic lethal (220). The PBR is found at high concentrations near outer/inner mitochondrial membrane contact sites, and several molecules may associate to form a channel (222). Additionally, PBR binds cholesterol in its cytosolic-facing C-terminus (223-225). Endozepine, also known as diazepam-binding inhibitor (DBI) due to its ability to displace diazepam binding to PBR, is the endogenous ligand for PBR (226). Inhibiting endozepine expression with antisense oligodeoxynucleotides prevents hCG-induced increases in progesterone production by MA-10 cells (227). Thus, endozepine may assist in cholesterol transport by causing conformational changes of PBR following second messenger stimulation. Coupled with the previously reported data indicating StAR import may not be required for cholesterol transfer to $p450_{scc}$, and therefore StAR's actions appear to occur at the outer mitochondrial membrane, the possibility of PBR creating a pore through which cholesterol passes is feasible. However, data on the PBR/endozepine system are still limited, and its role in cholesterol transport, if any, can still be debated. In one study, StAR transported cholesterol to $p450_{scc}$ in mitochondria that had proteins on the outer mitochondrial membrane removed by partial proteolysis with trypsin (228). Because PBR is localized to the outer mitochondrial

membrane, the finding that StAR transports cholesterol in the absence of outer membrane proteins would indicate that PBR is not involved in cholesterol transport. However, this is a doubtful conclusion based on a number of studies that indicate a vital role for PBR in steroidogenesis. Alternatively, there may be multiple mechanisms whereby StAR mediates cholesterol delivery to $p450_{scc}$, a notion that is believable considering it has been calculated that approximately 400 molecules of cholesterol are delivered per molecule of StAR before it is inactivated (229), so multiple mechanisms could allow increased efficiency of cholesterol delivery. In any case, it appears that while other proteins may be involved in cholesterol transport, StAR is the key player. Also, while PBR may be required, or at least assist, in cholesterol transport to $p450_{scc}$, it may not be a primary point of regulation in the acute control of steroidogenesis by PKA. Protein kinase-A phosphorylation sites exist in the C-terminal region of the rat, bovine and murine PBR sequences (reviewed in 226), and PBR has been demonstrated to be a substrate of PKA (230). However, the human PBR sequence does not have a PKA phosphorylation motif (226), and therefore phosphorylation of PBR by PKA may not occur in all species, and may not play a role in PKA-induced steroidogenesis.

Phosphorylation of StAR

Even before StAR was cloned, it was identified as a phosphoprotein (197-201, 203). Because the primary signaling pathway stimulated by tropic hormones is the cAMP/PKA pathway, PKA-induced phosphorylation of StAR and the subsequent biological effect is of considerable interest. Two putative motifs for PKA phosphorylation of StAR have been identified in murine, human, hamster, rat, bovine, ovine, and pig sequences

(reviewed in 231). These sites are serine residues 56/57 and 194/195 in the murine and human sequences respectively, with the single amino acid difference in location due to the absence of one residue in the N-terminus of the murine sequence (231). A direct correlation between the appearance of the phosphorylated form of StAR and steroidogenesis has been established (199). Therefore, the role of the putative PKA phosphorylation sites on the biological activity of StAR has been investigated. The serine residues at positions 57 and 195 in the human sequence were mutated to alanines, which both caused a reduction in the amount of ^{32}P incorporated into StAR (231), indicating that each site is phosphorylated by PKA in humans. The mutant constructs were transfected into Cos-1 cells, and mutating serine 57 had no effect on pregnenolone synthesis (231). This is not surprising considering that deletion of the first 62 amino acids of StAR has no effect on steroidogenesis (193). However, mutating serine 195 caused a 50% reduction in pregnenolone production (231). Mutation of the murine PKA phosphorylation sites produced similar results. Thus, it was concluded that PKA phosphorylation of StAR at serine 194/195 accounts, in part, for the immediate effects of cAMP on steroidogenesis (231). Because mutation of serine 194/195 did not completely block steroid production, StAR may retain some steroidogenic capacity in the absence of PKA phosphorylation, although PKA-induced phosphorylation clearly enhances StAR's cholesterol transferring capability. This may be especially important in periods of peak steroid production such as during maternal recognition of pregnancy. The immediate effect of cAMP, and subsequent StAR phosphorylation by PKA, is illustrated *in vivo* by the finding that serum concentrations of progesterone increase rapidly within 30 minutes following LH/hCG treatment in primates (232). Such a rapid response may be too fast to

be solely due to changes in protein expression, but more likely is because of post-translational modifications such as phosphorylation.

Steroidogenic acute regulatory protein is phosphorylated by other kinases including PKC (233) and casein kinase II (234). Human StAR expressed in non-treated Cos-1 cells exists in at least four species as determined by two-dimensional gel electrophoresis and western blotting, and two of these disappeared following alkaline phosphatase treatment (231) indicating that StAR can be phosphorylated under basal conditions. The importance of this basal phosphorylation, and which kinase(s) is responsible, is unknown. Phosphorylation of StAR by PKC could prove to be interesting as PKC is known to down-regulate steroidogenesis in luteal cells, and PKC is activated by the primary luteolytic hormone PGF2α in large cells (53). Three potential PKC phosphorylation sites are found in the mitochondrial targeting sequence of StAR, which may contribute to PKC-induced inhibition of steroid production by interfering with StAR transport to the mitochondria (55). The finding that the mitochondrial targeting sequence is not required for normal steroidogenesis (reviewed in 193) makes this hypothesis questionable. However, three additional PKC phosphorylation motifs are found in the 30 KDa amino acid sequence (mitochondrial associated form) of ovine StAR (8), so there is clearly a possible role for down-regulation of StAR activity via PKC-induced phosphorylation.

StAR Expression

Protein kinase-A and PKC, or the second messengers activating these kinases, also affect concentrations of StAR protein. Tropic hormone-stimulated steroidogenesis increases

StAR mRNA (235), and this effect can be mimicked with cAMP (236-240) even though the StAR promoter lacks a full consensus cAMP response element (241). The effect of cAMP to increase StAR transcription is not surprising considering cAMP's ability to stimulate steroidogenesis. Recently, it has been demonstrated that pharmacologic activation of PKC also increases the expression of StAR protein in leydig tumor cells, although the increase in StAR protein was not accompanied with a stimulation of steroid production because the newly translated protein was not phosphorylated by PKA (242). This indicates that increasing StAR protein alone is not sufficient to cause acute increases in steroid production, but PKA-induced phosphorylation of StAR is required as well.

StAR and PKA Regulation in Small and Large Steroidogenic Luteal Cells

One of the most baffling questions regarding luteal progesterone synthesis is why are large cells constitutively steroidogenic while small cells have lower basal production of progesterone that increases in response to hormonal stimulation? Considering the role of StAR and its phosphorylation by PKA in the acute control of steroidogenesis, the finding that large cells have high basal progesterone synthesis but do not respond to LH/hCG treatment with enhanced steroidogenesis despite the presence of LH receptors (16), or respond to non-hormonal increases in intracellular cAMP with increased progesterone production (53, 243), complicates a simple interpretation of the roles of StAR and PKA in this cell type. Increased expression of StAR protein in large versus small luteal cells may contribute to high basal progesterone synthesis by large cells (97). However, without a concomitant phosphorylation of StAR by PKA to fully activate it, it seems unlikely that higher concentrations of StAR could account for the dramatic difference in non-

stimulated progesterone secretion by the two cell types. One theory for the constitutive nature of progesterone production by large cells is that they have tonically active PKA (97, 243, reviewed in 8, 14).

To test the possibility of tonically active PKA in large luteal cells, Diaz *et al.* (2002) [97] treated purified large and small ovine luteal cells with PKI, a specific PKA inhibitor. Treatment of small luteal cells with PKI blocked forskolin-induced progesterone production as expected. Interestingly, PKI caused an approximately 50% inhibition in basal progesterone synthesis by large luteal cells. It was hypothesized that large cells may contain excess PKA catalytic subunits compared to regulatory subunits, and therefore have constitutive PKA-induced phosphorylation. Given what is known about the acute regulation of steroidogenesis by the cAMP/PKA pathway, and the fact that large cells secrete large quantities of progesterone under basal conditions, it seems reasonable that large cells do not respond to activation of the PKA pathway with increased progesterone production because PKA is already fully active in this cell type.

Summary

Progesterone is required for maintenance of pregnancy in all-mammalian species. The CL is the source of progesterone during estrous/menstrual cycles and early pregnancy. A large percentage of embryonic wastage that occurs during early pregnancy may be due to insufficient progesterone secretion by the CL. Therefore, understanding how progesterone is synthesized in the CL may lead to methods to decrease early embryonic loss, and development of more efficient assisted reproduction techniques. There are two

steroidogenic cell types in the CL, large cells that are constitutively steroidogenic, and small cells which have low basal progesterone synthesis that increases with activation of PKA. Understanding regulation of progesterone production in the two cell types is necessary to understanding progesterone secretion by the CL as a whole. Transport of cholesterol across the mitochondrial membrane is the rate-limiting step in steroidogenesis and is also the step most acutely influenced by second messenger systems. Therefore, it is a logical area to investigate differences in progesterone synthesis between large and small steroidogenic cells. The StAR protein is the best candidate for the factor that controls the acute regulation of cholesterol transport across the mitochondrial membrane in response to PKA. The mechanism of StAR action is unknown and may be mediated by PBR. Understanding the roles of StAR and PKA in cholesterol transport within the two steroidogenic cell types may lead to a better understanding of their differences in progesterone synthesis.

Chapter 2

Peripheral-Type Benzodiazepine Receptor (PBR) Aggregation and Absence of Steroidogenic Acute Regulatory Protein (StAR)/PBR Association in the Mitochondrial Membrane as Determined by Bioluminescence Resonance Energy Transfer (BRET)

Introduction

The transport of cholesterol to the mitochondria and across the mitochondrial membrane is the rate-limiting step in steroidogenesis and is the step most acutely influenced by second messengers (reviewed in 193). Steroidogenic acute regulatory protein (StAR) is believed to be responsible for the acute transport of cholesterol across the mitochondrial membrane (reviewed in 193). However, the mechanism of StAR-associated cholesterol transport to the inner mitochondrial membrane is unclear. Models attempting to explain the mechanism of StAR action have included: an intermembrane shuttle hypothesis, sterol desorption from one membrane to another, molten globule-induced cholesterol transport, or interactions with other proteins (reviewed in 244). Numerous studies have provided evidence that StAR import into the mitochondria may not be necessary for cholesterol delivery to the inner mitochondrial membrane (214-216). Therefore, other proteins may be involved in cholesterol transport from the outer to the inner

mitochondrial membrane. The peripheral-type benzodiazepine receptor (PBR) is an integral mitochondria membrane protein that has been implicated in steroidogenesis (221). Endozepine is the endogenous ligand for PBR (226), and inhibiting endozepine expression prevented hCG-induced increases in progesterone production by mouse leydig tumor cells (227). Therefore, StAR, PBR, and/or endozepine may interact resulting in cholesterol transport across the mitochondrial membrane.

West *et al.* (2001) tested the possibility of a StAR/PBR interaction by fusing the genes encoding ovine StAR and PBR with enhanced green fluorescent protein (GFP) and yellow fluorescent protein (YFP) respectively, and measured fluorescence resonance energy transfer (FRET) to determine if StAR and PBR associate at the mitochondrial membrane. They determined that the StAR-GFP and PBR-YFP fusion proteins were $\leq$ 70Å apart. It was hypothesized that StAR targets cholesterol to the outer mitochondrial membrane and transfers it to PBR for import across the inter-membrane space.

The goal of the current project was to confirm a StAR/PBR association and determine if this interaction is regulated by second messenger systems. Interactions between endozepine and StAR or PBR were also tested. Factors that activate protein kinase A (PKA) increase cholesterol transport and steroidogenesis, while activation of protein kinase C (PKC) inhibits PKA-stimulated steroidogenesis in ovine small luteal cells (53). Activation of the PKA second messenger pathway stimulates steroidogenesis by mouse Leydig tumor cells (MA-10) (246) similar to what occurs in ovine small luteal cells (53). Thus, it was hypothesized that PKA would have positive and PKC would have negative

effects on the StAR/PBR/endozepine interactions in MA-10 cells. Additionally, it has been suggested that PBR aggregates at the mitochondrial membrane to form a pore (246, 247). It is possible that StAR and PBR interact resulting in cholesterol transfer from StAR to the PBR pore, or that the StAR-cholesterol complex is imported into the mitochondria via a PBR pore.

Bioluminescence resonance energy transfer (BRET) was used to test for StAR/PBR/endozepine interactions and PBR aggregation. In BRET, renilla luciferase (RLuc) (donor molecule) hydrolyzes coelenterazine substrate which results in emission of blue light, differing from FRET that requires an external light source to excite the donor molecule. This blue light will excite humanized green fluorescent protein (hGFP) (acceptor molecule) if the molecules are in close proximity. In contrast to single cell FRET analysis (245), BRET is rapidly measured from a pool of cells in 96 or 384 well plates, and thus is better suited for analysis of a large number of experimental groups and to evaluate the effect of activation of second messenger pathways.

Materials and Methods

Materials

All cloning vectors and DeepBlueC® coelenterazine substrate were obtained from BioSignal Packard. All restriction enzymes and T4 DNA ligase used for DNA sub-cloning were purchased from MBI Fermentas Inc. Tissue culture supplies (plates, pipettes, etc.) were purchased from Sarstedt Inc., Dulbecco's Modified Eagles Medium

(DMEM) and antibiotic/antimycotic concentrate (containing penicillin G, streptomycin, and amphotericin B) were from Mediatech Inc., and fetal bovine serum (FBS) was from Gemini Bio-Products. PolyFect® transfection reagent, plasmid purification kits, and DNA agarose gel extraction kits were purchased from Qiagen Inc. White 96 well plates were from Life Science Products Inc. MitoTracker® Red CMXRos (MitoRed) was purchased from Molecular Probes Inc. Dibutyryl adenosine 3',5'-cyclic monophosphate (db-cAMP), phorbol 12-myristate 13-acetate (PMA), and 1-(2-Chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide (PK11195) were from Sigma-Aldrich.

Donor and Acceptor Fusion Proteins

The following plasmid constructs were created: StAR-hGFP, PBR-hGFP, endozepine-hGFP, StAR-RLuc, PBR-RLuc, endozepine-RLuc, and the mitochondrial targeting sequence of human cytochrome C oxidase (MitoTg)-RLuc. Because StAR contains an N-terminal mitochondrial targeting sequence (193) and the C-terminus of PBR is on the cytosolic side of the membrane (224), the RLuc or hGFP tags were placed at the C-terminus of StAR and PBR. The sequences for ovine StAR, PBR and endozepine were cloned into either pGFP-N1 or pGFP-N2 vectors, resulting in expression of the hGFP tag at the C-terminus of each protein. Additionally, the PBR sequence was cloned into pRLuc-N2, StAR into pRLuc-N1, and endozepine into pRLuc-C2 which places the RLuc tag at the N-terminus of endozepine. For all constructs except PBR-RLuc which utilized Hind III at the 5'end and StAR-RLuc that used BglII at the 5' end, EcoRI and BamHI at the 5' and 3' ends respectively were used for directional sub-cloning. Sequences corresponding to ovine StAR, PBR, or endozepine with the appropriate restriction

enzyme sites were generated either by polymerase chain reaction or restriction enzyme digest of plasmid constructs previously cloned in our laboratory. The presence of inserts and in-frame linkage to either RLuc or hGFP tags was confirmed by DNA sequence analysis.

Cell Culture and Transfection

Chinese hamster ovary (CHO), MA-10, and green African monkey kidney (Cos-7) cells were incubated in DMEM containing 5% heat-inactive FBS, 100 I.U./ml penicillin G, 100μg/ml streptomycin, and 250ng/ml amphotericin B at 37° C, 5% CO_2. Approximately 3.5×10^5 MA-10, CHO, or Cos-7 cells were transfected in 6-well plates using PolyFect® transfection reagent following manufacturer recommendations for Cos-7 cells. Medium was changed 24 hours post-transfection, and cells were analyzed between 42-54 hours post-transfection, except for time course assays. In some cases cells were treated with 5mM db-cAMP, 100nM PMA, or 5μg/ml PK11195 prior to BRET analysis. Energy transfer was then measured after a 4 hour treatment, or between 1-20 minutes after initiation of treatment.

BRET Assay

Medium was removed and plates washed with phosphate buffered saline (PBS). Cells were scraped in 200μl of Dulbecco's-PBS (DPBS), and 40μl added in triplicate to white 96 well plates. A 1mM stock of DeepBlueC® coelenterazine substrate was prepared in absolute ethanol, diluted 1:40 in DPBS, and 10μl added (5μM). Immediately after substrate addition, dual-luminescence was measured in a Mithras LB 940 plate reader

(Berthold Technologies) with a 400 ± 35nm filter for RLuc, 515 ± 10nm for hGFP, and a 1 second reading time for each filter. The equation for calculating BRET ratio was: (avg. hGFP transfected – avg. hGFP non-transfected) / (avg. RLuc transfected – avg. RLuc non-transfected).

Progesterone Radioimmunoassay

Concentrations of progesterone in media were determined by radioimmunoassay (248). The inter-assay coefficient of variation (CV) was 20.9, 7.2, and 6.2% for the low, medium, and high concentration quality controls respectively. The intra-assay CV averaged 5.2% and 10.4% at 20% and 80% respectively on the theoretical standard curve for two assays. Sensitivity of the assays averaged 1.16 picograms per tube.

Confocal Laser Scanning Microscopy (CLSM)

Trafficking to the mitochondrial membrane was tested for StAR, PBR, and endozepine proteins fused to hGFP. Mouse leydig tumor cells were transfected as described previously in glass-bottom culture dishes. Mitochondria were labeled with MitoRed, a derivative of X-rosamine that specifically localizes to mitochondria (249). Approximately 42 hours post-transfection, cells were incubated at 37°C for 15 min with 50nM MitoRed in normal growth medium. Medium was removed, cells washed three times with PBS, and fresh medium added. Cells were incubated under normal growth conditions an additional 5-6 hours. Cells were fixed using 3.7% paraformaldehyde for 15 min at 37°C, washed three times with PBS, and stored in the dark at 4°C until analysis. Cells were visualized with a Zeiss LSM510 confocal laser scanning microscope using 488nm and

543nm excitation and 505-530nm and 585nm emission for green and red fluorescence respectively.

Statistical Analyses

Data were analyzed using analysis of variance in the statistical analysis system (SAS). Means for PBR/StAR, PBR/endozepine, and endozepine/StAR interactions were analyzed using Dunnett's t-test comparing against the empty hGFP controls. Means for concentrations of progesterone in media were analyzed with t-tests comparing each treatment to the non-treated control. Means for PBR aggregation and effects of treatments, dose responses, and time course analysis were analyzed using Tukey's Studentized Range test.

Results

Data obtained from MA-10 and CHO cells were similar and therefore were combined for analysis. There was no evidence of StAR/PBR, endozepine/StAR, or PBR/endozepine associations in either cell type (Figure 2.1). Because MA-10 and CHO cells are steroidogenic, it was hypothesized that endogenously expressed StAR, PBR and endozepine may have prevented detection of energy transfer between the fusion protein constructs. Therefore, interactions between fusion proteins were furthered studied in the non-steroidogenic Cos-7 cell line. Similar to MA-10 and CHO cells, no energy transfer was observed between any of the fusion protein combinations while the positive control plasmid (BRET+), which produces an RLuc:hGFP fusion protein, caused a significant

increase in the 515nm/410nm ratio (Figure 2.2). No StAR/PBR interactions were detected in MA-10 cells after increasing the ratio of acceptor:donor plasmids up to 10:1 StAR-hGFP:PBR-RLuc (data not shown). Furthermore, no interactions were detected in MA-10 cells between StAR-RLuc and PBR-hGFP at ratios ranging from 5:1 to 1:7 RLuc:hGFP (data not shown). Additionally, bacterial and mammalian two-hybrid assays were employed to test for protein/protein interactions. No StAR/PBR, endozepine/StAR, or PBR/endozepine interactions were detected using either of these methods (data not shown).

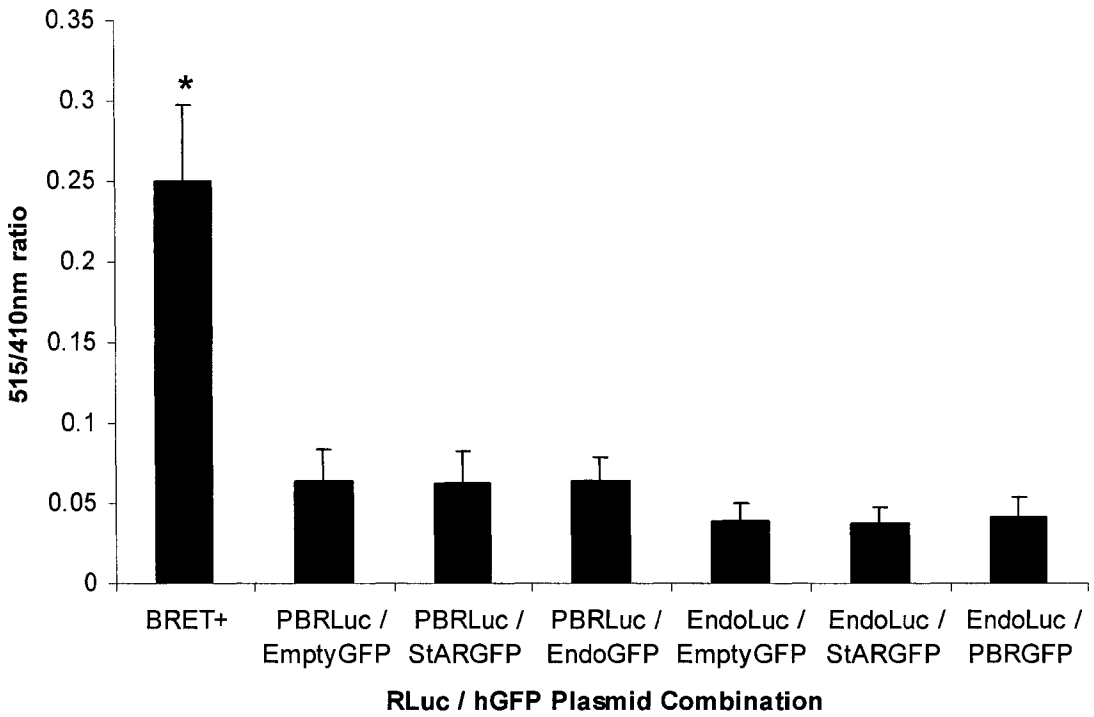


Figure 2.1: Energy Transfer in MA-10 and CHO cells. Average of four transfections performed on different days (two in each cell type). The positive control (BRET+) produces an RLuc:hGFP fusion protein. Negative controls are PBRLuc / EmptyGFP and EndoLuc / EmptyGFP. Error bars indicate one standard error of the mean (SEM), asterisk denotes significant ($p < .05$) difference from either negative control.

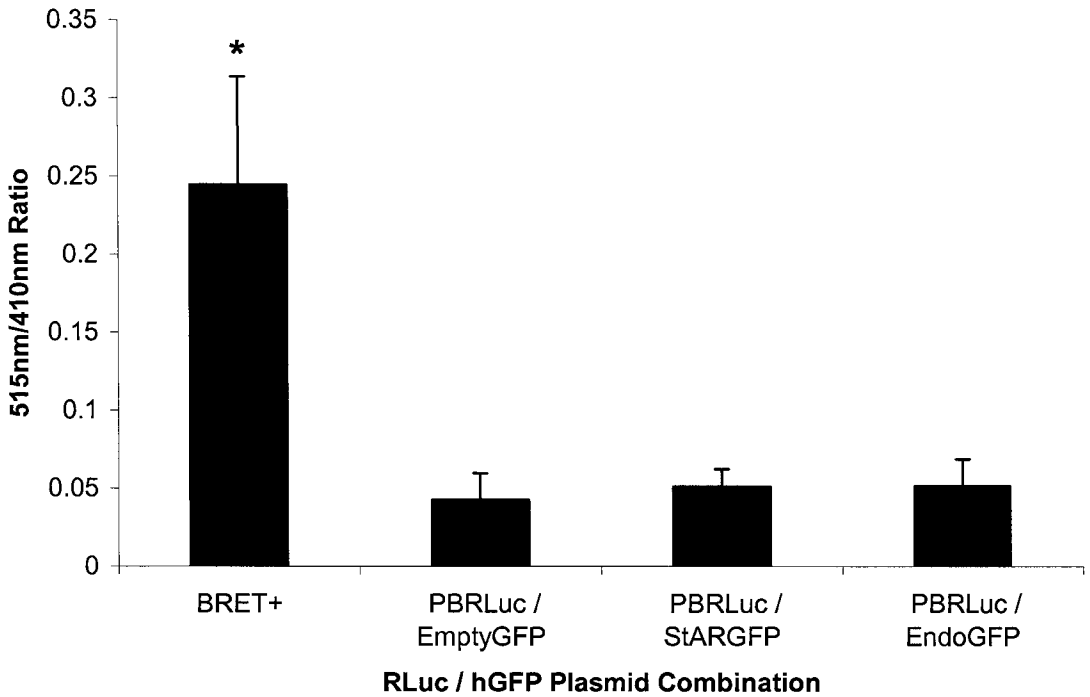


Figure 2.2: Energy transfer in Cos-7 cells. Average of three transfections performed on different days. Error bars indicate one SEM, asterisk denotes significant ($p < .05$) difference from the PBRLuc / EmptyGFP negative control.

The possibility that PBR aggregates at the mitochondrial membrane was also tested. In Cos-7 cells, transfecting a 1:1 ratio of PBR-RLuc/PBR-hGFP plasmids resulted in a significant ($p< .05$) increase in the BRET ratio compared to PBR-RLuc/emptyGFP (Figure 2.3). Additionally, the mitochondrial targeting sequence of human cytochrome C oxidase (MitoTg) was fused to renilla luciferase and used as a control for non-specific energy transfer that may occur as result of RLuc and hGFP molecules being targeted to the mitochondrial membrane. Energy transfer between MitoTg-RLuc/PBR-hGFP was not different than PBR-RLuc/emptyGFP (Figure 2.3). Next, the dose dependency (increasing the ratio of acceptor:donor plasmids transfected) of PBR aggregation was tested.

Increasing the ratio of PBR-hGFP:PBR-RLuc plasmids resulted in an increase in energy transfer that was significantly ($p < .05$) higher at 7:1 or 10:1 ratios compared to a 1:1 ratio (Figure 2.4), while there was no change in non-specific energy transfer between PBR-RLuc/emptyGFP or MitoTg-RLuc/PBR-hGFP with increasing hGFP:RLuc ratios. Therefore, a 7:1 ratio of PBR-hGFP:PBR-RLuc plasmids was used in additional experiments. Time course assays were performed, and there was a significant ($p < .05$) increase in energy transfer by 24 hours compared to 12 hours post-transfection (Figure 2.5) in PBR-RLuc/PBR-hGFP transfected cells. There were no significant changes in energy transfer at time points measured between 24 and 48 hours for PBR-RLuc/PBR-hGFP transfected cells, and no significant differences between any time points measured for BRET+ or PBR-RLuc/empty-hGFP transfected cells.

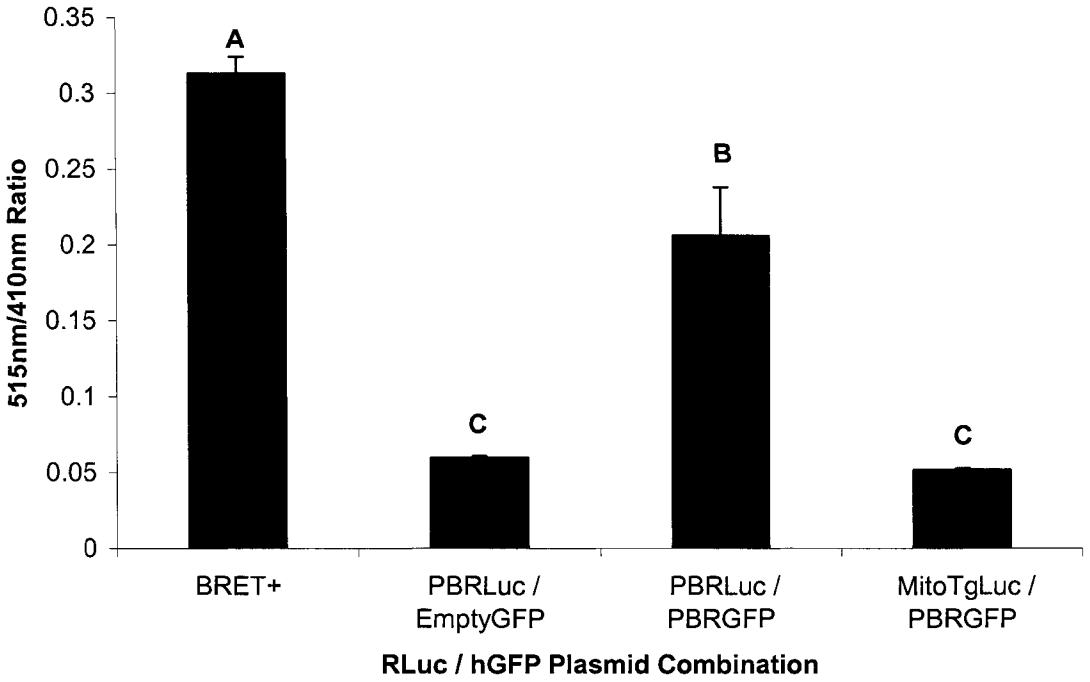


Figure 2.3: Energy transfer in Cos-7 cells. Average of two transfections performed on different days. Error bars indicate one SEM, columns with different letters are significantly different ($p < .05$).

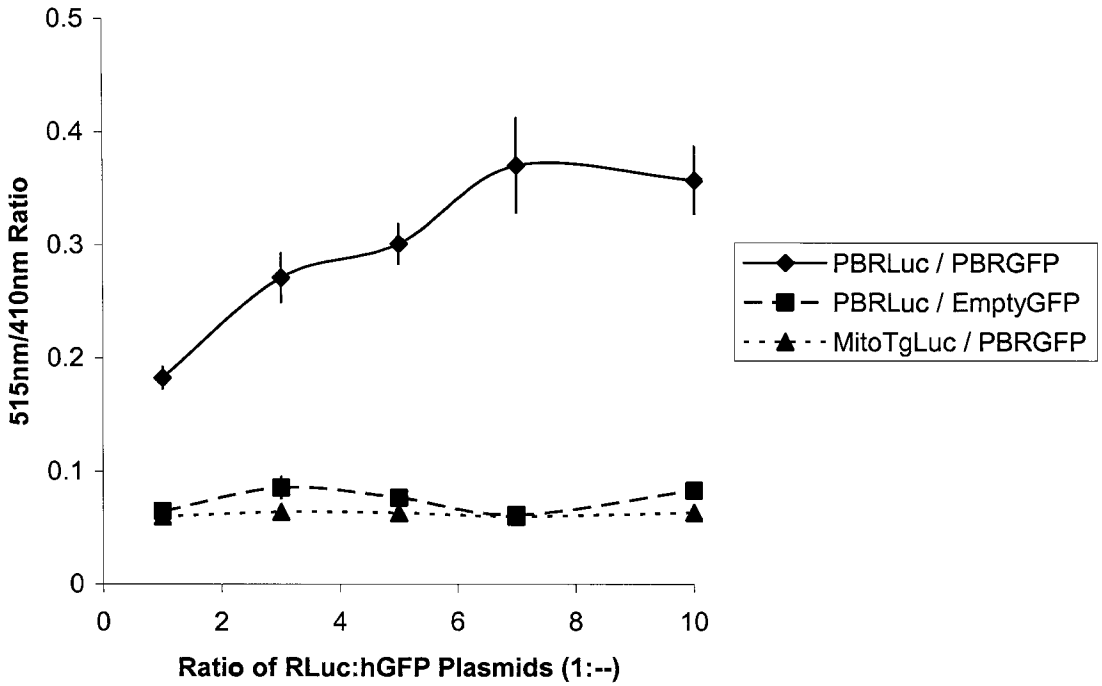


Figure 2.4: Dose dependency of energy transfer in MA-10 cells. Analyzed at 48 hours post-transfection. Average of 2 independent transfections, error bars indicate ±SEM.

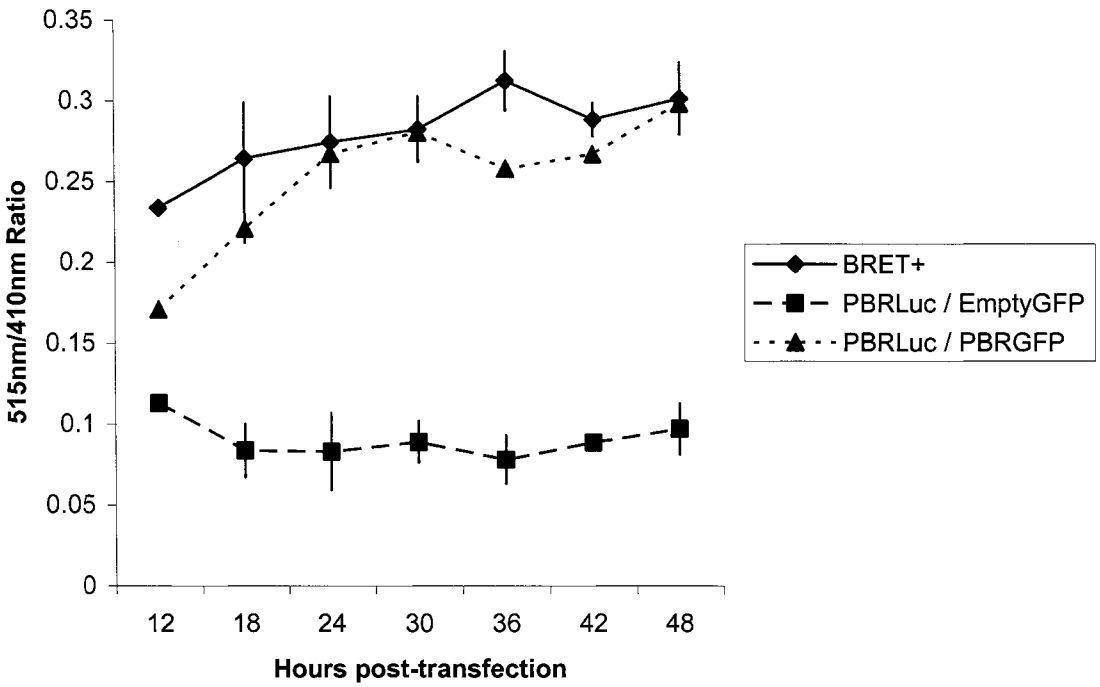


Figure 2.5: Time course of energy transfer in MA-10 cells. Average of 2 assays performed in different weeks with a 7:1 ratio of hGFP:RLuc transfected, error bars indicate ± one SEM.

The effects of second messengers or ligand binding on PBR aggregation was evaluated in MA-10 cells. There was no effect of treatment with db-cAMP, PMA, or the PBR ligand PK11195 when compared to non-treated cells with either long (4 hours) (Figure 2.6) or short (< 20 min) (data not shown) term treatment. Also, there was no evidence of a change in aggregation caused by db-cAMP, PMA, or PK11195 when a lower hGFP:RLuc ratio (1:1) was transfected (data not shown). Concentrations of progesterone in media from PBR-RLuc/PBR-hGFP transfected cells were analyzed to verify treatment effects. Media was collected from 2 of the transfections that received 4 hour treatments with db-cAMP, PMA, or PK11195. The treatments induced the expected biological effect on steroidogenesis as concentrations of progesterone were: 1.3 ± 0.3ng/ml for non-treated,

757.5 ± 8.1ng/ml ($p < .01$) with db-cAMP treatment, 1.0 ± 0.5 for PMA-treated, and 6.2 ± 1.1ng/ml ($p < .05$) with PK11195-treated cells.

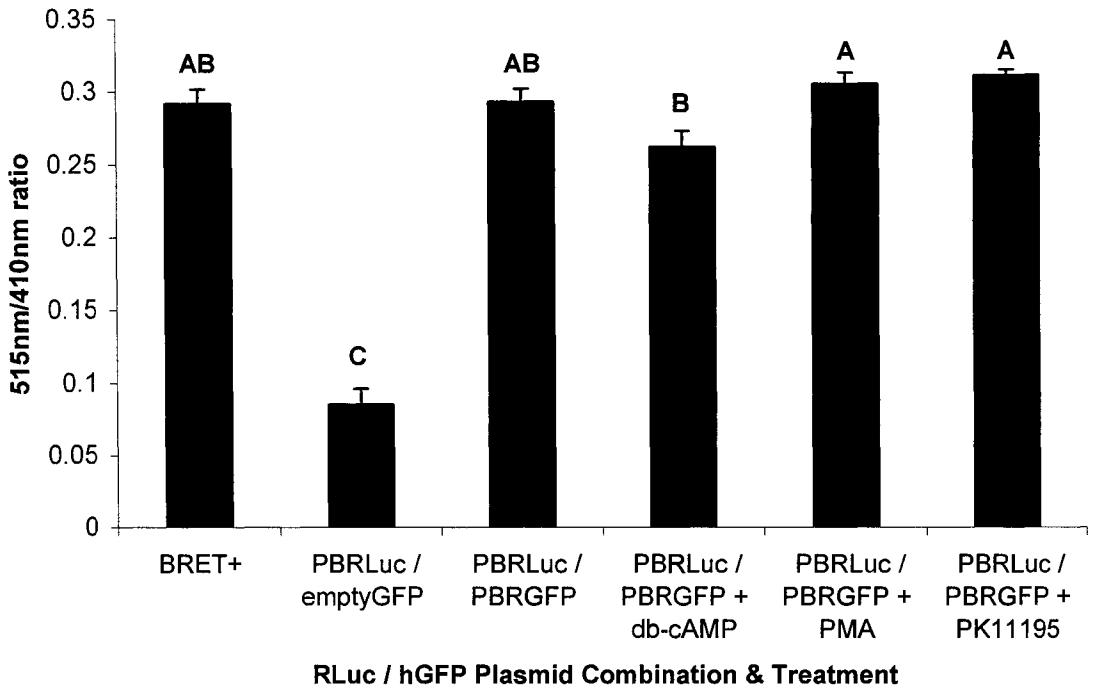


Figure 2.6: Effects of various treatments on PBR/PBR energy transfer in MA-10 cells. Average of 4 transfections performed on different days, error bars indicate one SEM. Columns without common letters are significantly different ($p < .05$).

Confocal laser scanning microscopy (CLSM) was used to test for mitochondrial localization of hGFP fusion proteins. There was no evidence of co-localization between hGFP and MitoRed in cells transfected with hGFP cloning vector (Figure 2.7 A). Co-localization was observed between PBR-hGFP or StAR-hGFP and MitoRed (Figure 2.7 C & D), but endozepine-hGFP (Figure 2.7 B) did not appear to be trafficked to the mitochondria.

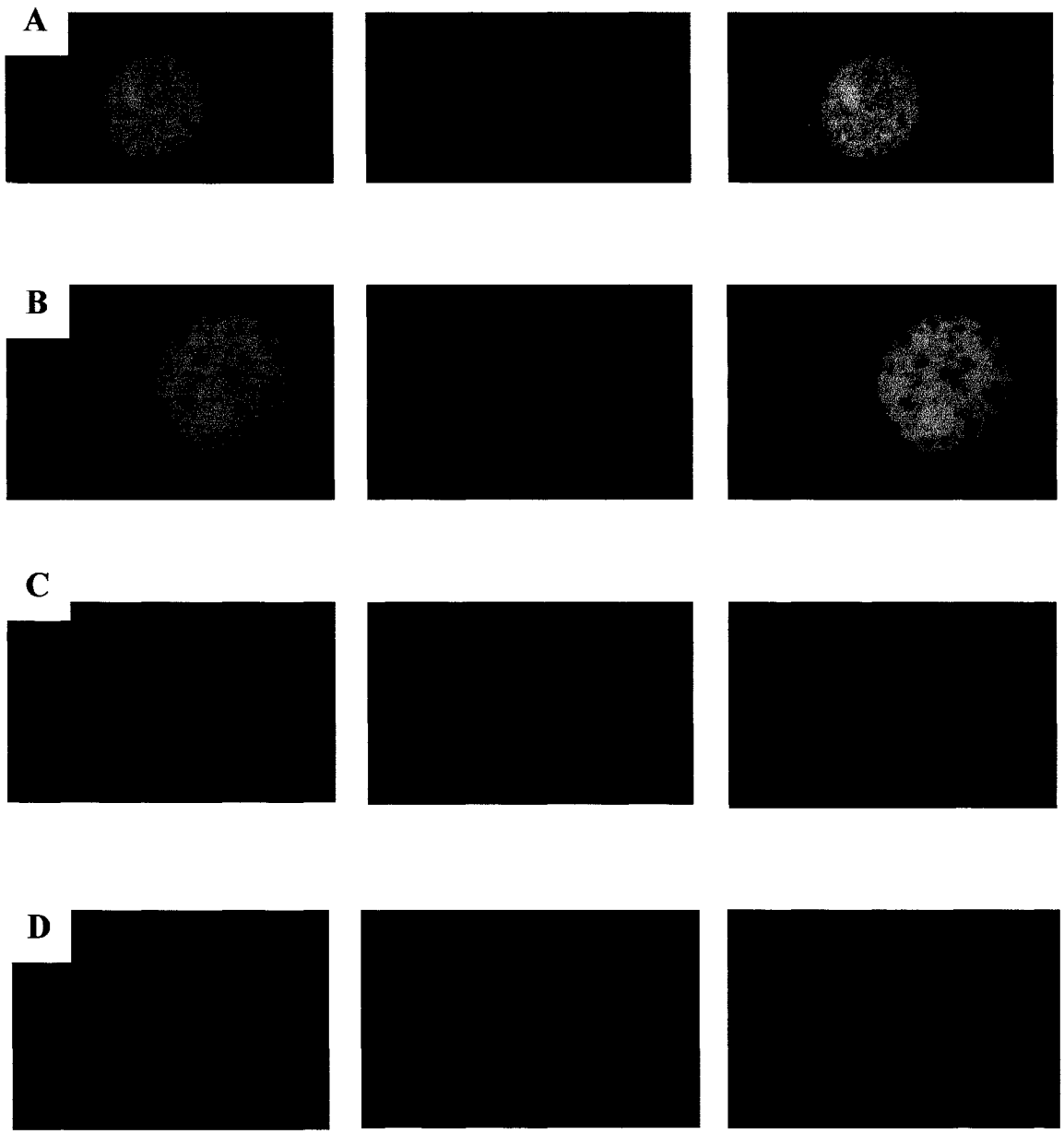


Figure 2.7: Confocal laser scanning microscopy images of hGFP fusion proteins. For each row, images are hGFP only, MitoRed only, and overlay from left to right respectively. Row A is a cell transfected with hGFP cloning vector, B is endozepine-hGFP, C is PBR-hGFP, and D is StAR-hGFP.

Discussion

There was no evidence obtained in the present studies that StAR and PBR interact. While this conflicts with previous findings (245), it can not be ruled out that there is a transient proximity or association between StAR and PBR that is more easily detected in single-cell FRET measurements. If this is the case, this would explain why no association was detected in the current study because BRET analysis was performed on pools of cells. Therefore, a transient association might not be detected as the data represent the mean of a large number of cells. Increasing the ratio of hGFP:RLuc plasmids transfected may increase energy transfer observed between interacting proteins, but even when a 10:1 ratio of StAR-hGFP:PBR-RLuc was transfected, no specific energy transfer was detected. It was possible that using StAR-RLuc to donate to a more stable PBR-hGFP molecule might increase the probability of detecting a StAR/PBR association if one exists. However, no energy transfer from StAR-RLuc to PBR-hGFP was detected over a broad range of StAR-RLuc:PBR-hGFP ratios. While a transient proximity between StAR and PBR can not be ruled out, it appears that there is not a stable interaction or association between StAR and PBR.

It was surprising that a PBR and endozepine interaction was not detected. This result may be explained by the results after confocal imaging of the hGFP constructs. As seen in figure 2.7 C, PBR-hGFP is clearly localized to the mitochondria. However, in figure 2.7 B, there is no evidence that endozepine-hGFP has been trafficked to the mitochondria. Therefore, it appears that the endozepine construct may not be biologically active. This may not be surprising since endozepine is an 87 amino acid protein (250) while hGFP has 238 amino acids (251). Thus, the presence of the much larger hGFP protein may have

influenced the localization and biological activity of endozepine. Also, in figure 2.7 D, StAR-hGFP is co-localized with MitoRed, indicating that the StAR construct is present in the mitochondria as expected. Thus, the BRET data indicating there is no StAR/PBR association is not due to altered trafficking of the StAR or PBR fusion proteins.

Data reported in this study provide strong evidence that PBR aggregates at the mitochondrial membrane. Aggregation of PBR was observed in both steroidogenic (MA-10) and non-steroidogenic (Cos-7) cells. However, there was no evidence that PBR aggregation is affected by activation of PKA (db-cAMP treatment), PKC (PMA treatment), or by ligand binding (PK11195) (Figure 2.6). It is possible that energy transfer with a 7:1 hGFP:RLuc ratio was near the upper limit of sensitivity. Therefore, any increase in aggregation induced by treatments would not be detected. However, even at a 1:1 ratio of hGFP:RLuc, no change in aggregation was observed with any treatment. Also, PKC which is believed to inhibit cholesterol transport may be expected to decrease PBR aggregation, but there was no evidence of any effect.

The first evidence of PBR aggregation came from studies involving gold immunolabeled PBR molecules, which indicated that native PBR is found in clusters of 4-6 molecules (247). Data reported herein support that finding. Later, it was discovered that reactive oxygen species induced the formation of covalent PBR polymers by stimulating the formation of dityrosine bonds (252). It was hypothesized that PBR polymers represent the functional unit in cholesterol transport. Delavoie *et al.* (2003) reported that PBR in non-stimulated MA-10 cells was found primarily in its monomeric form, which contrasts

with results in the present study that indicate PBR aggregates under basal conditions in MA-10 cells. The positive control vector used in the present report produces an RLuc:hGFP fusion protein to insure energy transfer, and energy transfer from the PBR-RLuc:PBR-hGFP transfected cells was not significantly different than the positive control in MA-10 cells (Figure 2.6). This strongly suggests the presence of a PBR-hGFP molecule in the immediate vicinity of every PBR-RLuc molecule in non-stimulated MA-10 cells.

It has been reported that human chorionic gonadotropin (hCG) treatment of MA-10 cells caused an increase in the number of PBR molecules per aggregate (246). Considering that hCG activates the cAMP/PKA pathway in MA-10 cells (246), a similar effect on PBR aggregation would be expected with db-cAMP treatment. In this study there was no effect induced by db-cAMP on PBR aggregation even though db-cAMP caused a >500 fold increase in progesterone synthesis. Using BRET analysis, it appears that PBR aggregation occurs under basal conditions and is not affected by activation of PKA, PKC, or by PK11195 binding. Because PBR is an integral mitochondria membrane protein that forms aggregates, it is possible that PBR clusters may form a pore in the mitochondrial membrane which allows cholesterol delivery to the inner mitochondrial membrane. The cholesterol may be free or bound to other transport proteins (i.e. StAR). While these pores may be important structures aiding in cholesterol transport, BRET analysis provides no evidence that they are regulated as part of the acute control of steroidogenesis, or that they are linked with StAR's transport of cholesterol.

Chapter 3

Development of an Assay to Quantify Concentrations and Relative Phosphorylation States of the Steroidogenic Acute Regulatory (StAR) Protein

Introduction

Studies investigating phosphorylation of endogenously expressed StAR have been performed primarily using two-dimensional (2-D) gel electrophoresis followed by western blotting with antibodies against either StAR protein or phosphorylated protein (244, 253-254). While 2-D electrophoresis and western blotting allow visualization of different StAR species, it is a time-consuming technique that is not practical for analysis of a large number of samples and is difficult to precisely quantitate. At least two species of StAR with a molecular weight of approximately 30 KDa but different isoelectric points are identified by 2-D electrophoresis/immunoblotting (244, 253-254). The number of StAR species increases following stimulation by tropic hormones (244, 253), indicating there may be different forms of phosphorylated StAR. However, few studies have attempted to quantify relative changes in StAR phosphorylation.

To better understand regulation of StAR activity in steroidogenic cells, determining both the concentration and the phosphorylation state of StAR are important. Therefore, a

method that quantifies concentration and relative phosphorylation of endogenously expressed StAR protein is needed. Additionally, it would be desirable to have an assay that is very sensitive to reduce the quantities of tissues or purified cell preparations required for experiments. The present study focused on development of a sandwich, enzyme-linked immunosorbent assay (ELISA) that would quantify concentration and relative phosphorylation state of StAR in steroidogenic cells.

Materials and Methods

Materials

Tissue culture supplies (plates, pipettes, conicals) were obtained from Sarstedt Inc., fetal bovine serum (FBS) was from Gemini Bio-Products, and Dulbecco's Modified Eagles Medium (DMEM) and antibiotic/antimycotic concentrate (containing penicillin G, streptomycin, and amphotericin B) were purchased from Mediatech Incorporated. Forskolin was from Calbiochem/Novabiochem Corp., and phorbol 12-myristate 13-acetate (PMA) from Sigma-Aldrich. Supplies used for protein purification included: dialysis membrane (6-8000 molecular weight cut-off) from Spectrum Labs, Centricon-10 filter devices and .2 micron syringe filters from Millipore Corp., isopropyl β-D-1-thiogalactopyranoside (IPTG) from Gold Biotechnology Inc., Affi-Gel 10 gel from Bio-Rad Inc., and nickel-nitrilotriacetic acid (Nickel-NTA) agarose purchased from Qiagen. Supplies for ELISA's included black 96-well tissue culture-treated plates from Corning Inc., calf intestinal alkaline phosphatase (CIAP) from MBI Fermentas, Supersignal™ ELISA Pico chemiluminescent substrate, and Supersignal™ ELISA Femto

chemiluminescent substrate purchased from Pierce Biotechnology. For immunoprecipitations, protein A-agarose was from Invitrogen Life Sciences, protein G-agarose and X-ray film were from Pierce Biotechnology, and enhanced chemiluminescent substrate (ECL) was purchased from Amersham Biosciences.

Antibodies, Antibody Production and Purification

The modified sandwich ELISA uses three antibodies; 1.) a StAR-specific capture antibody, 2.) a secondary antibody that is either StAR-specific (for determining StAR protein concentration) or phosphoprotein-specific (for measuring phosphorylation state), and 3.) a horse radish peroxidase (HRP)-conjugated antibody specific for immunoglobulin (IgG) of the species used to produce the secondary antibody. An affinity-purified rabbit anti-StAR polyclonal antibody (Affinity Bioreagents, #PA1-560) was used as the capture antibody. The secondary StAR-specific antibody for quantifying concentration of StAR protein was produced by immunizing a goat with a BSA-conjugated peptide that corresponded to amino acids 115-127 of the ovine StAR sequence. Peptide synthesis, purification, and immunization were performed by Affinity Bioreagents (ABR). A mouse monoclonal anti-serine/threonine phosphoprotein antibody (BD Biosciences, #612549) served as secondary antibody for the StAR phosphoprotein ELISA. Antibodies conjugated to HRP were rabbit anti-goat IgG (Santa Cruz Biotechnology Inc., #sc-2768) and goat anti-murine IgG_1 pre-adsorbed with human IgG (Santa Cruz, #sc-2969). An HRP-conjugated goat anti-rabbit IgG (Santa Cruz, #sc-2004) antibody was used for western analysis.

The goat anti-StAR serum received from ABR was antigen affinity-purified by adding 4.5mg of peptide to 4.5ml of dimethyl sulfoxide (DMSO), and mixing with 3 ml (settled bed volume) of Affi-Gel 10 for 2.5 hours at room temperature. Unreacted sites in the gel matrix were blocked by adding 300µl of 1M ethanolamine (in DMSO), and incubating for 1 hour at room temperature. The gel was washed sequentially with 15ml each of Tris-buffered saline (TBS), wash buffer #1 (1M NaCl, 20mM Tris-HCL, pH 7.4, 0.2% Triton X-100), elution buffer (100mM glycine, 150mM NaCl, 10% dioxane (v/v), pH 2.25), and TBS again until pH of the flow-through reached approximately 7.4. Anti-serum was heat-inactivated at 55°C for 30 min, and filtered through a 0.2 micron syringe filter. Fifteen ml of anti-serum were diluted 1:1 with TBS and incubated with the affinity gel overnight at 4°C. The mixture was then added to a column, and the affinity gel was washed sequentially with 60ml of TBS, 60ml of wash buffer #1, 15ml elution buffer pH 4.0, and 40ml TBS. Antibody was eluted in 1ml fractions of elution buffer (pH 2.25), collected in tubes containing 100µl of 1M Tris pH 8.8 to immediately neutralize pH. The optical density (OD) at 280nm was determined, and fractions containing peak absorbance were pooled, dialyzed against TBS overnight at 4°C, and concentrated by centrifugation at 3,000g through a Centricon-10 filter (10,000 molecular weight cut-off). Protein concentration was determined (Pierce Biotech.) following manufacturer recommendations. The affinity-purified antibody was diluted 1:1 in freezing buffer (80% glycerol, TBS, 0.2% NaN_3) and stored in small aliquots at -20°C. The affinity column was reused multiple times by removing non-eluted antibody with 15ml of stripping buffer (6M guanidine hydrochloride in TBS). The column was stored at 4°C in TBS with 0.2% NaN_3.

Recombinant StAR Protein Production

The cDNA encoding the 37 KDa form of ovine StAR was cloned into the pRSET A expression vector, and transformed into BL21(DE3) pLysS *E. coli* cells (expression vector and cells from Invitrogen Life Sciences). Expression from the pRSET A vector results in 6 consecutive histidine residues (6x-His) at the N-terminus of StAR, and allows StAR purification due to the high affinity of the 6x-His tag for nickel (255). Three starter cultures of 20ml each were grown from glycerol stocks in LB medium containing 200μg/ml ampicillin and 35μg/ml chloramphenicol, and cultured overnight at 37°C. The cultures were then transferred to 1.35 liters of LB medium and cultured an additional 2 hours at 37°C until the OD at 600nm of the cultures was approximately 0.5. Expression of recombinant StAR protein was induced with 2mM IPTG. Cells were incubated with IPTG 4 hours at 37°C, harvested by centrifugation at 5,000g for 15 min, and pelleted cells stored overnight at -70°C. The next day, cells were lysed in 12.5ml of 8M urea, 10mM Tris-HCL, 25mM imidazole, pH 8.0, and incubated at room temperature for 1 hour. The lysate was cleared by centrifugation at 10,000g for 30 min at 4°C. The supernatant was collected, and 1.5ml of 50% Nickel-NTA agarose added followed by incubation at room temperature for 1 hour. The Nickel-NTA agarose was pelleted by centrifugation at 5,000g for 5 min, washed twice with 4ml of 8M urea, 10mM Tris-HCL, pH 6.3, and twice with 4ml of 300mM NaCl, 20mM imidazole, pH 8.0. The recombinant StAR protein was eluted in 4 fractions (500μl each) of 300mM NaCl, 250mM imidazole, pH 8.0. Eluates were pooled and dialyzed against TBS overnight at 4°C. Following dialysis, the eluate was concentrated by centrifugation at 3,000g in Centricon-10 filter

devices for 30 min at 4°C. Protein concentration was determined, then 25µl of freezing buffer were added per 75µl of eluate and small aliquots stored at -20°C. Additionally, 40µl of the pooled eluate collected prior to the dialysis step, was resolved by 12% de-naturing poly-acrylamide gel electrophoresis (SDS-PAGE), and the gel stained with Coomassie Brilliant Blue. Protein bands were visualized using a NucleoTech UV gel box, and the purity of the band corresponding to StAR was determined by Gel Expert software analysis.

Modified Sandwich ELISA for StAR Protein

All volumes are 100µl unless otherwise noted, and all washing steps were performed by filling plate wells four times with TBS. Standards were developed from recombinant StAR protein (0-300ng/well), and quality controls (QC's) consisted of 15µg of small luteal cell lysate with nothing (low QC), 25ng (medium QC), or 75ng (high QC) of recombinant StAR protein added. All standards, QC's, unknown samples, and antibodies were diluted in TBS with 1% (w/v) non-fat dry milk (NFDM) unless otherwise noted. The capture antibody (rabbit anti-StAR) was diluted in TBS to 2.5µg/ml, and coated to wells of black 96-well plates by passive adsorption for approximately 24 hours at 4°C. The capture antibody solution was removed, and wells were washed, followed by blocking non-bound sites with 300µl of TBS with 5% NFDM for 4 hours at room temperature. Recombinant StAR protein standards, QC's, and unknowns were added to triplicate wells and incubated at 37°C for 3 hours. Un-bound proteins were decanted, the plate washed, and 75ng/well of secondary antibody (goat anti-StAR) added and incubated overnight at 4°C. After removal of the secondary antibody and washing, 75ng/well of

HRP-conjugated rabbit anti-goat IgG were added and incubated at room temperature for 4 hours. Following the final wash, SuperSignal™ ELISA Pico chemiluminescent substrate was added. Luminescence measurements were taken in a Mithras LB 940 plate reader (Berthold Technologies Inc.) programmed to shake for 1 min followed by measuring luminescence with a 425 ±20nm filter and reading for 0.2 seconds/well. Additionally, the plate was exposed to X-ray film for 2-5 min to provide visual verification of machine readings.

Control wells were included that were not coated with capture antibody and did not receive sample, but were incubated with secondary and HRP-conjugated antibody. The average luminescence of these control wells was subtracted from the average of each standard, QC, or unknown sample to control for background luminescence. The concentration of StAR protein in each QC or unknown sample was determined by comparison to the standard curve in each assay.

Modified Sandwich ELISA for StAR Phosphorylation

The procedure to measure StAR phosphorylation was similar to the ELISA that measured StAR protein with the following exceptions: concentration of the capture antibody was 3μg/ml, the secondary antibody was the anti-serine/threonine phosphoprotein antibody, the HRP-conjugated antibody was goat anti-mouse IgG_1 pre-absorbed with human IgG, and substrate for detection was SuperSignal™ ELISA Femto chemiluminescent substrate. The amount of protein analyzed was adjusted based on results from the ELISA for determining concentration of StAR protein so that 20ng per well of StAR were added.

Mixed luteal cells treated in serum-free DMEM with 10μM forskolin and 100nM PMA for 2 hours at 37°C, 5% CO_2 in air were used to create phosphorylation standards. Cells were collected in 500μl homogenization buffer [250mM sucrose, 10mM Tris-HCL, pH 7.4, 10mM EDTA, 1mM sodium orthovanadate, protease inhibitor cocktail (Roche Diagnostics Inc.) and phosphatase inhibitor cocktail 1 (Sigma-Aldrich) added at manufacturer recommended concentrations]. Cells on ice were lysed by sonication using a Branson sonifier at 4 pulses of 10 seconds each. Lysates were boiled at 95°C for 4 min immediately following sonication. Concentration of StAR protein on a per microgram protein basis, was determined using the assay for StAR protein.

To create a standard curve for StAR phosphorylation, the forskolin/PMA-treated mixed luteal cell lysate was considered the maximally phosphorylated StAR standard, and bacterially-produced recombinant StAR protein, which does not have post-translational modifications, was used for the non-phosphorylated standard. The maximally phosphorylated and non-phosphorylated stocks were mixed to produce a set of standards at 100, 75, 50, 25, and 0% StAR phosphorylation. Additionally, three QC's were included consisting of mixed luteal cells treated with nothing, 10μM forskolin, or 100nM PMA. Wells receiving all three antibodies but no sample were included and their average luminescence was subtracted to correct for background. The percentage of StAR phosphorylation in each QC was determined using the background-corrected average luminescence compared to a standard curve generated from standards run in four separate assays.

Corpora Lutea Preparation

Corpora lutea (CL) were collected from ewes on day 11 post-ovulation to provide protein for various validation procedures. Corpora lutea were dissected into eighths, and one-eighth of a CL from 3 different ewes was homogenized in 3ml homogenization buffer. Homogenates were cleared by centrifugation (5,000g for 5 min), and protein concentration determined as described previously. Aliquots were made and frozen at -20°C until used.

Western Analysis

For quantitative western analysis, standards (3-300ng/lane of recombinant StAR) and unknowns were resolved by 12% SDS-PAGE, and transferred to a nitrocellulose membrane. Unbound sites were blocked with TBS containing 0.1% Tween 20 and 2% (w/v) bovine serum albumin (BSA) at room temperature for one hour. Membranes were probed with an IgG-purified rabbit anti-StAR serum (1:1500) produced previously in our laboratory against amino acids 123-134 of the ovine StAR sequence. The anti-StAR serum was diluted in TBS containing 0.1% Tween 20 and 1% BSA, and incubated with the membranes overnight at 4°C. Membranes were washed with one change of TBS and 3 changes of TBS containing 0.1% Tween 20. An HRP-conjugated secondary antibody against rabbit IgG was diluted 1:3500 and incubated with the membranes for 1.5 hours at room temperature. Membranes were washed as before, and blots developed with enhanced chemiluminescent substrate for 5 min, exposed to X-ray film for 1-3 min, and

scanned using a STORM imager. Intensity of bands detected by STORM imaging was determined with ImageQuant 5.2 analysis.

Alkaline Phosphatase Digestion

De-phosphorylation of samples utilized calf intestinal alkaline phosphatase (CIAP) incubated with lysate at 1 unit of CIAP per 3μg of total protein. The reaction proceeded at 37°C for 75 min, followed by heat inactivation of CIAP at 65°C for 20 min.

Immunoprecipitations

Immunoprecipitation (IP) followed by western analysis was performed using mouse leydig tumor cells (MA-10). Cells were plated at 3×10^6 cells in 100mm tissue culture plates in DMEM containing 5% heat-inactivated FBS, 100 I.U./ml penicillin G, 100μg/ml streptomycin, and 250ng/ml amphotericin B, and cultured at 37°C, 5% CO_2 in air. One day after plating, medium was replaced with serum-free DMEM and cells cultured 2 hours. Medium was replaced again and 10μM forskolin, or 100nM PMA, was added and cells cultured 4 hours. At the end of the treatment, medium was aspirated, cells washed twice with TBS and collected in 400μl homogenization buffer. Homogenates were prepared as described previously.

The IP reactions utilized 200μg of protein, 3μg of antibody, 50μl of protein A-agarose, 25μl of protein G-agarose for IP reactions utilizing mouse IgG, and TBS to a 500μl final volume. Different reactions were performed using either the rabbit anti-StAR or the anti-serine/threonine phosphoprotein antibodies for IP. Negative control reactions were

included that did not have antibody added. The reactions were incubated overnight at 4°C on an end-over-end shaker. The protein A and G-agarose were pelleted by centrifugation at 5,000g for 3 min at 4°C. The rabbit anti-StAR IP reactions were washed three times with 500µl of RIPA lysis buffer (50mM Tris-HCL, pH 7.4, 150mM NaCL, 1mM EDTA, 5µg/ml aprotinin, 5µg/ml leupeptin, 0.1% Triton x-100, 1% sodium deoxycholate, and 0.1% SDS), and the anti-serine/threonine phosphoprotein IP reactions were washed three times with 500µl of TBS. After washing, 40µl of TBS were added, and tubes boiled at 95°C for 3 min. Agarose was pelleted by centrifugation at 5,000g and the supernatant collected. Samples were subjected to western analysis as described previously using the rabbit anti-StAR capture antibody (8µg) or goat anti-StAR serum (1:1500) to probe the membranes.

Statistical Analyses

Analysis of variance (ANOVA) in the statistical analysis system (SAS) was used to calculate standard errors of the mean (SEM) for each standard and QC in both modified sandwich ELISA assays, and inter-assay and intra-assay coefficients of variation (CV) for the QC's. Differences between means were analyzed using t-tests.

Results

Validation of Modified Sandwich ELISA for StAR Protein

Purified recombinant StAR was used as the standard. Bands at approximately 30 and 40 KDa were detected by Coomassie Brilliant Blue stain in the purified preparation, and the 40 KDa band corresponded to StAR (37 KDa StAR + poly-histidine tag) as determined

by western analysis using two different StAR-specific antibodies (Figure 3.1). A standard curve for StAR protein pooled from 4 assays had a linearity of $R^2 = .99$ over a range of 0-300ng of StAR protein per well (Figure 3.2). Intra-assay CV's were calculated using the raw luminescence values for each QC from its respective triplicate wells, and averaged 17.4, 15.5, and 13.9% in three assays for the low, medium, and high QC's respectively. The concentration of StAR protein was calculated in each QC by averaging the luminescence values from triplicate wells, subtracting the average assay background, and comparing to the standard curve generated with each assay (Figure 3.3). Inter-assay CV's were determined using the calculated concentration of StAR protein in each QC (expressed as ng of StAR/well) from each of 3 assays and were 12.7, 13.5, and 4.8% for low, medium, and high QC's respectively. Intra-assay variation was higher because it was calculated from the luminescence of individual wells whereas inter-assay variation was determined from a mean value of triplicate wells.

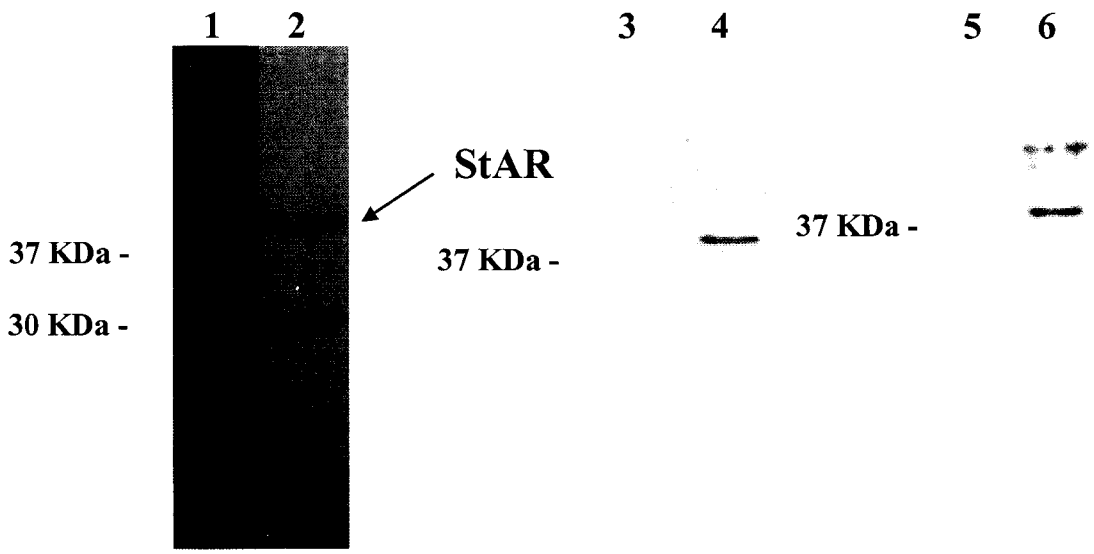


Figure 3.1: Bands detected in purified recombinant StAR preparation by Coomassie Brilliant Blue stain (Lanes 1-2), western analysis using a goat anti-StAR antibody (lanes 3-4), or western analysis using a rabbit anti-StAR antibody (lanes 5-6). Lanes 1, 3, and 5 is the molecular weight marker.

There were 2 bands present in the eluate, but only the band positioned slightly above the 37 KDa marker corresponds to StAR protein as determined by western analysis.

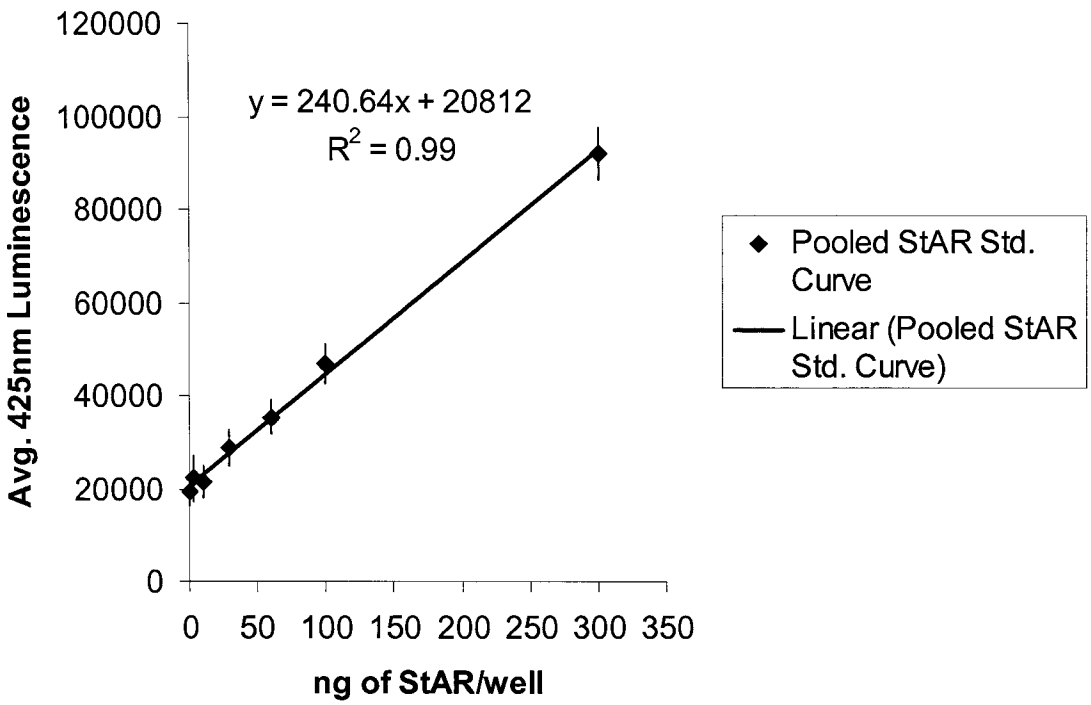


Figure 3.2: Standard curve for StAR protein pooled from 4 assays performed on different days. Error bars indicate ± one standard error of the mean (SEM).

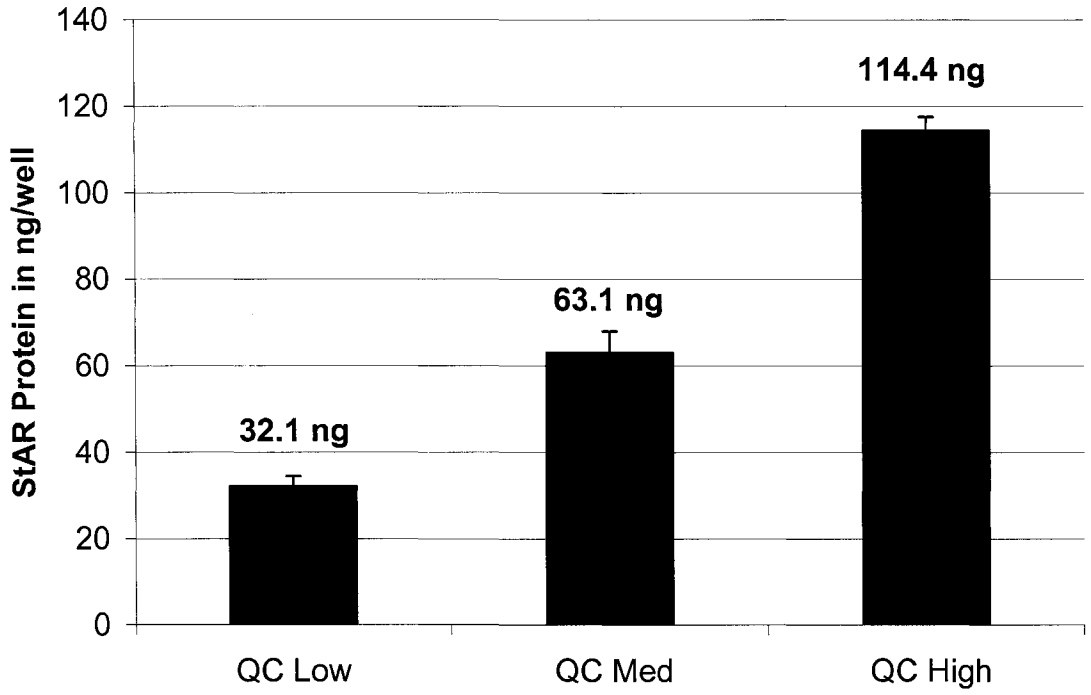


Figure 3.3: Calculated concentration of StAR protein in quality controls (QC). Averaged from 3 assays, error bar indicates one SEM.

Specificity of the ELISA for quantifying StAR protein was determined by checking parallelism of curves developed from recombinant StAR protein that was used as the standard, and StAR protein detected in various quantities of CL homogenate. Recombinant StAR (0-300ng/well) and CL homogenate (10, 30, and 60μg/well) were analyzed in 3 independent assays. The quantity of StAR in each amount of CL homogenate was calculated from the standard curve developed with each assay. When super-imposing the two curves on a linear scale (Figure 3.4), the curves were considered parallel if the SEM for each quantity of CL homogenate on both the X and Y axis's crossed or touched the linear trendline produced by the recombinant StAR standards. Using this method, the curves were considered parallel.

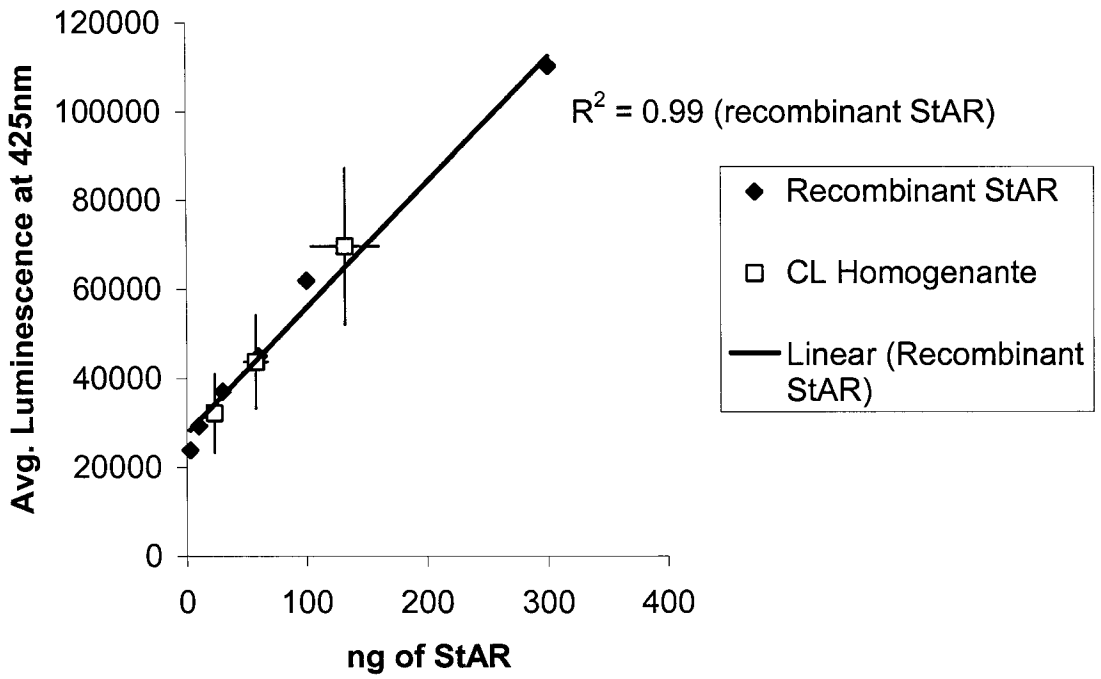


Figure 3.4: Recombinant StAR standards and increasing quantities of CL homogenate plotted on a linear curve. Error bars indicate ±SEM for the CL homogenate on both Y axis (luminescence units) and X axis (calculated StAR concentration).

Accuracy of the ELISA was tested against quantitative western analysis. The concentration of StAR protein was quantified by both methodologies in 10μg of CL homogenate with 0, 10, or 30ng of recombinant StAR added. The predicted concentration of StAR was determined by averaging the amounts calculated in 10μg of CL homogenate by both methods, and adding the indicated amounts of recombinant protein. The two methods were very similar in their calculated concentration of StAR for CL homogenate with 0, 10, or 30ng recombinant protein added. When plotting the two methods together (Figure 3.5), the slope is 1.02 with an R^2 of 0.99. Therefore, the calculated concentrations of StAR protein determined by ELISA were similar to those obtained by a different methodology (Figures 3.6 and 3.7), and therefore substantiates the ELISA method.

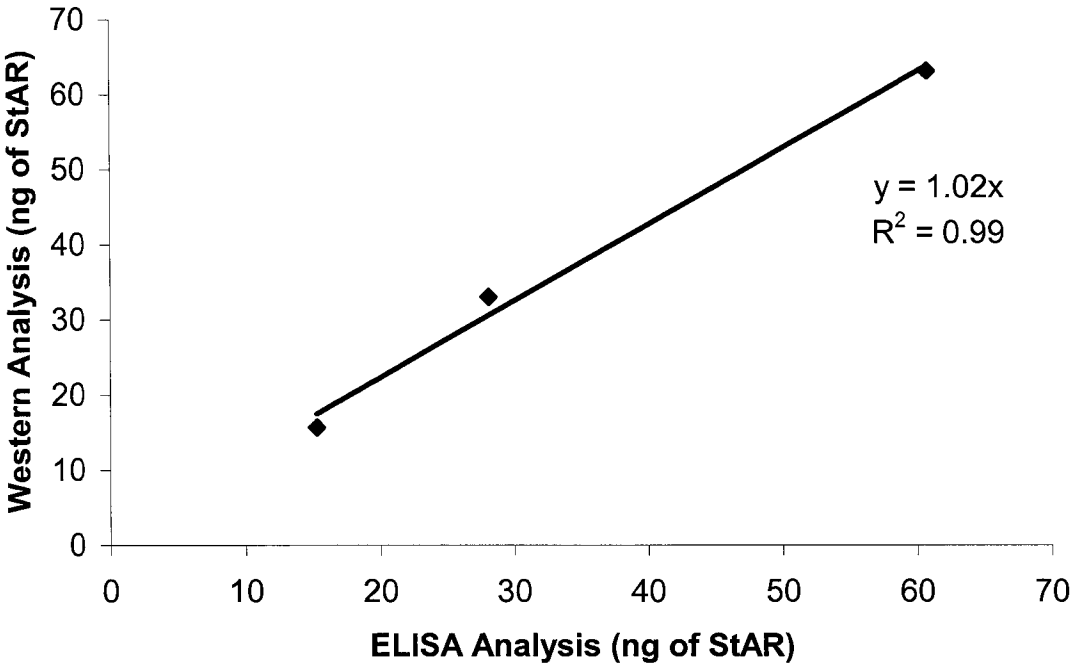


Figure 3.5: Comparison of StAR quantification by western analysis (Y axis) and ELISA (X axis). Values calculated from 3 independent assays for each method.

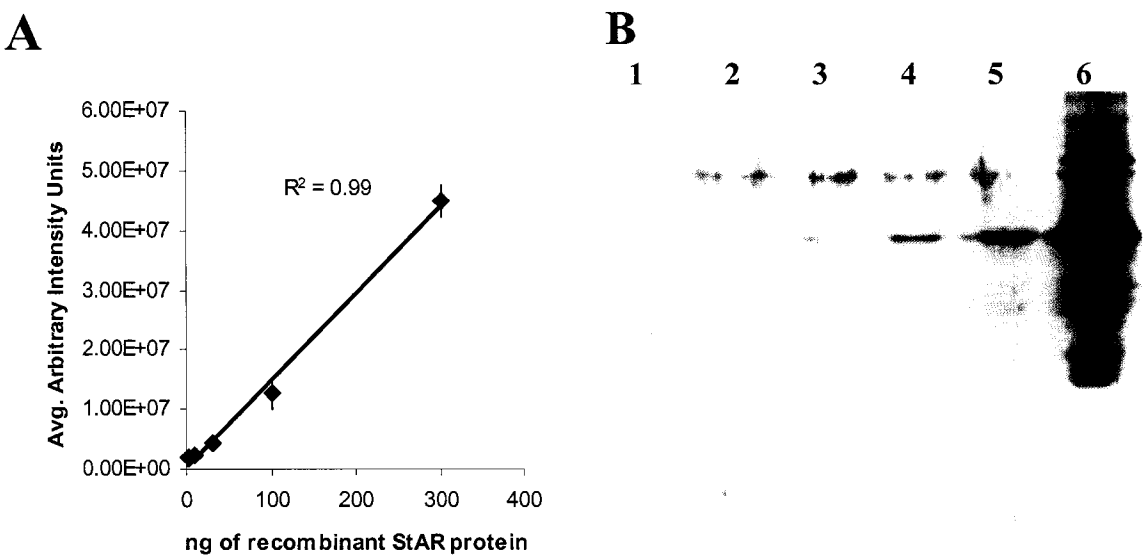


Figure 3.6: Standard curve for StAR protein developed from 3 membranes analyzed on different days (A) and representative membrane with recombinant StAR standards (B). In B, Lane 1 is the molecular weight marker, lanes 2-6 are 3, 10, 30, 100, and 300ng recombinant protein respectively.

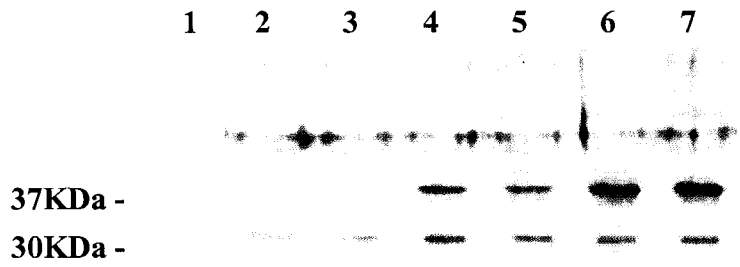


Figure 3.7: Representative western of CL homogenate with increasing amounts of recombinant StAR. Lane 1 is molecular weight marker, lanes 2-3 are 10μg CL, lanes 4-5 have 10ng recomb. StAR added, and lanes 6-7 have 30ng added. The bands at ~37 KDa are recombinant StAR and the bands at ~30 KDa are StAR from the CL homogenate. The 37 and 30 KDa bands were combined for quantification.

Validation of Modified Sandwich ELISA to Quantify Relative StAR Phosphorylation

Because StAR is potentially phosphorylated by PKA and PKC (55), StAR protein from forskolin/PMA-treated mixed luteal cells was considered the maximally phosphorylated standard because theoretically it would be phosphorylated at all possible residues. Mixed luteal cells treated with nothing (low), forskolin only (medium), or PMA only (high) were used as quality controls. To produce standards for StAR phosphorylation, recombinant StAR protein, which is not phosphorylated, was added to the maximally phosphorylated standard while holding the amount of total StAR protein constant. The average background-corrected luminescence for each standard from each assay was used to produce a single standard curve that all unknowns and QC's were compared against. The standard curve for relative StAR phosphorylation pooled from 4 assays had a linearity of $R^2 = .97$ (Figure 3.8). Intra-assay CV was determined using the raw luminescence values for the triplicate wells of each QC run with each assay. The mixed

luteal cell QC's (Figure 3.9) had average intra-assay variations of 14.7, 4.1, and 15.2% for the low, medium and high QC's respectively. Additionally, the percentages of StAR phosphorylation in QC's were calculated by comparing the average background-corrected luminescence from each assay against the pooled standard curve. Inter-assay variation was 5.5, 12.2, and 24% for low, medium and high QC's respectively. Theoretically, the calculated relative phosphorylation in the QC's should equal 100%. To determine the additive effect, the difference in phosphorylation between the medium QC (forskolin-treated cells) and low QC (non-treated cells) is added to the phosphorylation calculated in the high QC (PMA-treated cells) (Figure 3.9). This is because presumably forskolin and PMA are causing phosphorylation at different sites, but the value for the low QC is already included in both the medium and high QC values (low QC represents basal phosphorylation, treatments are basal plus treatment effect). By this method, the additive value is 109.1%.

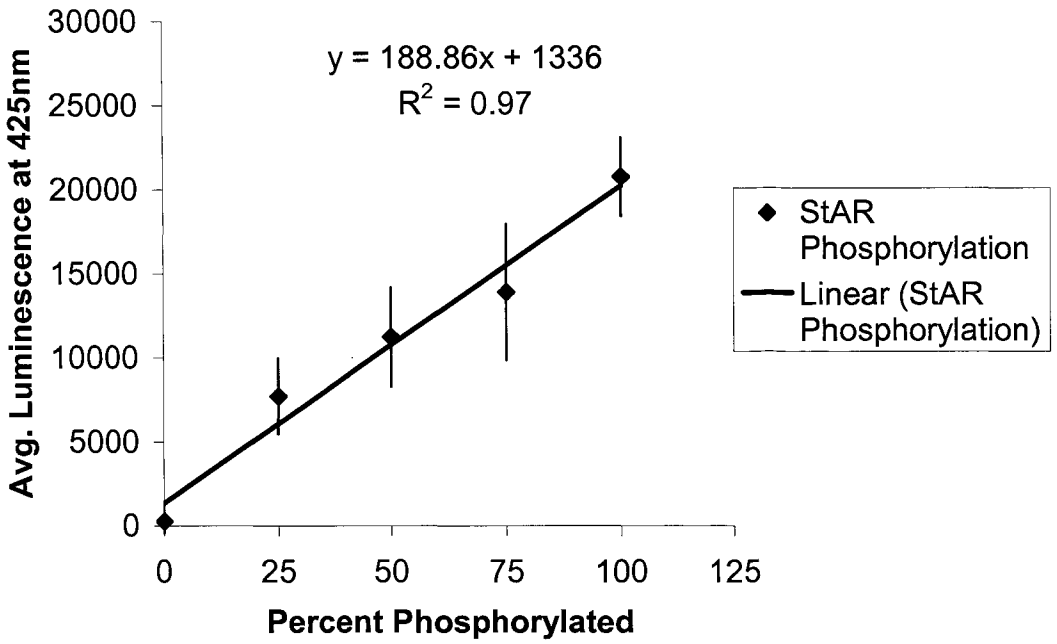


Figure 3.8: Standard curve for relative StAR phosphorylation. Averaged from 4 independent assays, error bars indicate ±SEM.

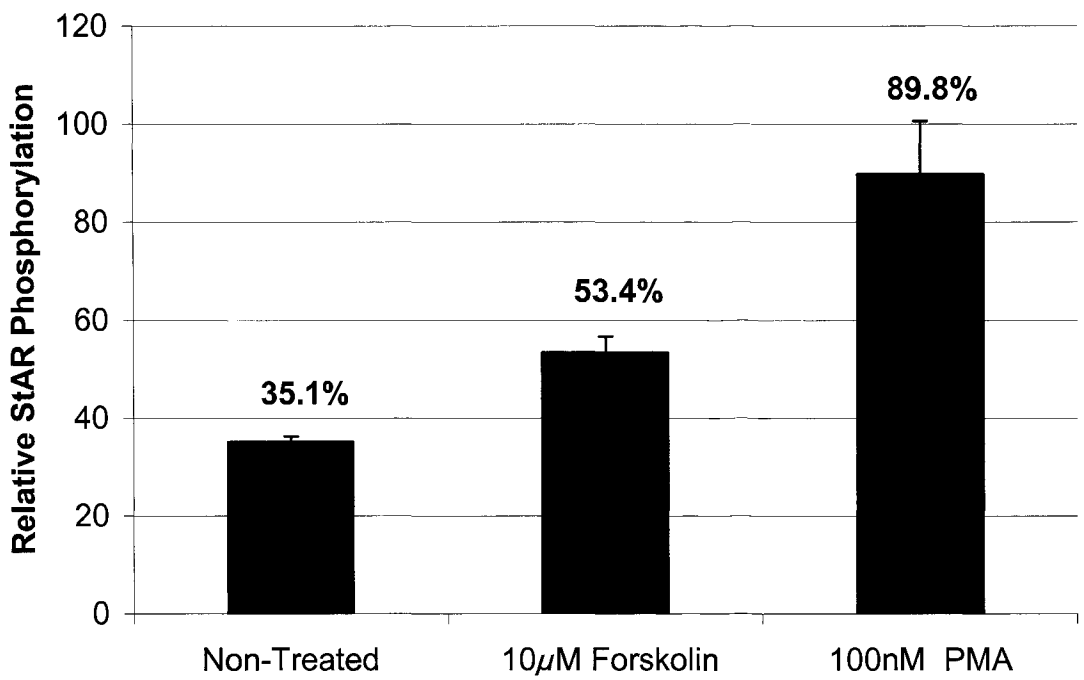


Figure 3.9: Relative StAR phosphorylation in mixed luteal cell QC's. Expressed as percent phosphorylation relative to standards produced from mixed luteal cells treated with forskolin and PMA (Figure 3.8). Average of 4 assays, error bars indicate +SEM.

Specificity of the assay was demonstrated by two methods. First, alkaline phosphatase sensitivity was used to demonstrate specificity of the anti-serine/threonine phosphoprotein antibody for phosphorylated residues. As seen in Figure 3.10, treatment with alkaline phosphatase caused an approximately 90% decrease in luminescence with the StAR phosphorylation ELISA but did not affect luminescence values obtained with the ELISA to quantify concentration of StAR protein. Specificity was further validated by development of the standards for relative StAR phosphorylation. Recombinant StAR protein caused a dose-dependant inhibition of phosphoprotein detected in the forskolin/PMA-treated mixed luteal cells down to control levels (Figure 3.8).

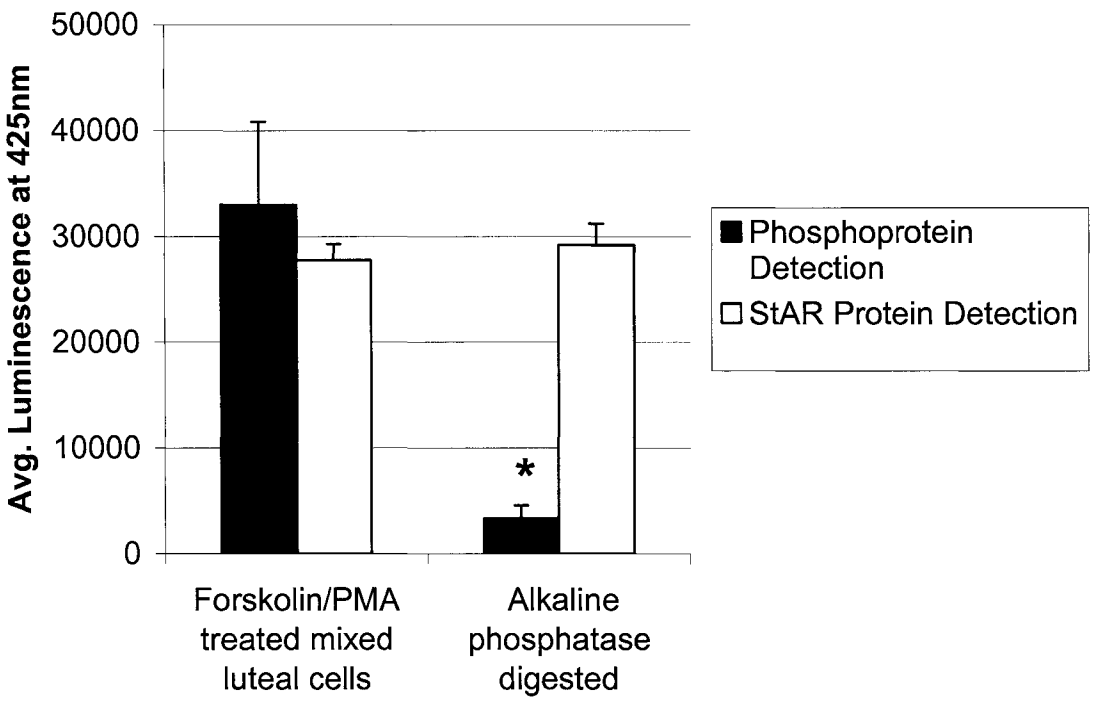


Figure 3.10: Detection of phosphoprotein and StAR protein in forskolin/PMA-treated mixed luteal cells with and without calf alkaline phosphatase digestion. Average of 3 assays for phosphoprotein and 2 assays for StAR protein. Error bars indicate +SEM, asterisk denotes significant decrease (p<

.05) in phosphoprotein detection. This is a different preparation of mixed luteal cells than what was used to make the standard curve.

Immunoprecipitations (IP) were utilized to further demonstrate specificity of the ELISA for quantifying StAR phosphorylation, and to test its accuracy. Whole cell lysates of MA-10 cells treated with either PMA (100nM) or forskolin (10μM) were used for analysis. Additionally, a portion of the lysate from PMA-treated cells was de-phosphorylated with alkaline phosphatase and used in an IP reaction, and a negative control consisting of forskolin-treated lysate without antibody in the IP reaction was included. Using the anti-serine/threonine phosphoprotein antibody to IP, both rabbit (data not shown) and goat anti-StAR (Figure 3.11) antibodies detected bands at ~30 KDa by western analysis in the forskolin and PMA IP samples. Alkaline phosphatase treatment of the lysate from PMA-treated cells caused a decrease in band intensity down to control levels when the anti-serine/threonine phosphoprotein antibody was used in the IP reaction. Additionally, the rabbit anti-StAR antibody was used in an IP reaction with the same lysates, and samples were analyzed by western using the goat anti-StAR antibody. Bands of similar intensity at ~30 KDa were detected in the forskolin, PMA, and alkaline phosphatase treated PMA IP samples (Figure 3.11).

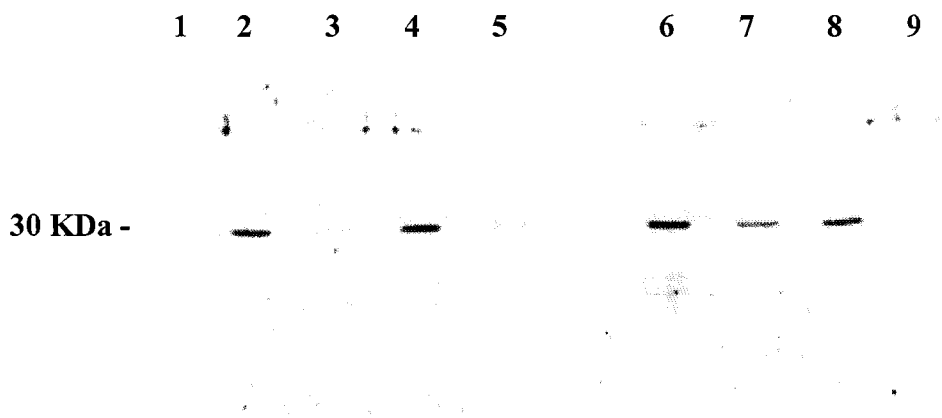


Figure 3.11: Western using goat anti-StAR serum. Lane 1 is the molecular weight marker, lanes 2-5 are IP samples using the anti-serine/threonine phosphoprotein antibody in the IP reaction. Lane 2 is lysate from PMA-treated cells, lane 3 is alkaline phosphatase-treated lysate from PMA-treated cells, lane 4 is lysate from forskolin-treated cells, and lane 5 is lysate from forskolin-treated cells without antibody in the IP reaction. Lanes 6-9 are IP samples using the rabbit anti-StAR antibody in the IP reaction, with lysates in the same order as lanes 2-5.

Discussion

This report describes a novel method for quantifying the concentration and relative phosphorylation state of StAR in biological samples. The standard curve for quantifying concentration of StAR protein has a linear range of at least 3-300ng of StAR protein per well. Inter and intra-assay variation of the three QC's was between 5-17% in all cases. Parallelism between recombinant StAR standards and endogenous StAR in CL homogenates was demonstrated, and quantifying StAR by ELISA produces results similar to quantitative western analysis.

The ELISA for quantifying relative phosphorylation of StAR had a linear standard curve and the three QC's had intra and inter-assay variations between 4-15% in nearly all cases. Quantification of phosphorylation could be more precise if the actual number of phosphorylated residues per molecule of StAR in the 100% standard was determined. Because the 100% standard was made from mixed luteal cells treated with forskolin and PMA, theoretically the QC's that were also mixed luteal cells treated with nothing, forskolin only, or PMA only should have additive phosphorylation values of 100%. The additive value was 109.1%, which is close to the expected value. Thus, these data demonstrate an efficient method for standardizing phosphorylation.

Specificity of the ELISA for relative StAR phosphorylation was demonstrated via alkaline phosphatase sensitivity and inhibition of phosphoprotein detection in mixed luteal cells with recombinant StAR protein. Alkaline phosphatase sensitivity demonstrates that phosphorylated residues were specifically being detected. The alkaline phosphatase treated mixed luteal cells had an average luminescence value only slightly higher than background, but approximately 90% lower than the same lysate that was not treated with alkaline phosphatase indicating that nearly complete de-phosphorylation had been achieved. However, this method only provides evidence that phosphorylated residues are being quantified, not necessarily phosphorylated residues on the StAR protein. Possibly, the capture antibody could cross-react with other proteins that are phosphorylated, or if complete blocking is not achieved, other phosphorylated proteins could bind to the plate wells. Inhibition of phosphoprotein detection in mixed luteal cells by purified recombinant StAR protein indicates that phosphorylated residues on the StAR protein are specifically being quantified. Bacterially-produced recombinant StAR is not phosphorylated. Recombinant StAR protein caused a dose-dependant inhibition of phosphoprotein detected in the forskolin/PMA-treated mixed luteal cells down to control levels, indicating that virtually all luminescence above background is due to specific binding of the anti-serine/threonine phosphoprotein antibody to phosphorylated residues on the StAR protein.

Immunoprecipitations provided some indication of the accuracy of the ELISA for relative StAR phosphorylation, as well as further demonstrating specificity of the assay. First, in

IP samples using the anti-serine/threonine phosphoprotein antibody, bands corresponding to the approximate molecular weight of StAR were detected by western using two different StAR-specific antibodies. This demonstrates that the anti-serine/threonine phosphoprotein antibody used in the ELISA binds to phosphorylated StAR. The fact that alkaline phosphatase treatment inhibited immunoprecipitation of StAR protein when the anti-serine/threonine phosphoprotein antibody was used to IP, but not when the anti-StAR antibody was used in the IP reaction, corroborates with the results obtained by ELISA in figure 3.10. This demonstrates that differences in the phosphorylation state per molecule of StAR were being quantified, and not changes due to varying quantities of StAR protein. It is worth noting that the phosphorylation detected in the low QC (non-treated cells) is most likely due to basal kinase activity and not assay background as non-treated MA-10 cells subjected to IP with the anti-serine/threonine antibody also yielded a band at ~30 KDa detected by both rabbit and goat anti-StAR antibodies (data not shown), indicating that there is basal phosphorylation of StAR. Finally, in regards to comparing the ELISA with immunoprecipitation, the IP reactions used 200μg of MA-10 protein, while the standards and QC's in the modified sandwich ELISA for StAR phosphorylation used approximately 20ng of StAR protein per well. Because StAR accounts for approximately 0.1-0.5% of soluble protein, this corresponds to 4-20μg of total protein analyzed in triplicate. Thus, the ELISA for relative StAR phosphorylation requires much less protein compared to immunoprecipitations.

In theory, the ELISA's described here that quantified either the concentration of StAR protein or its relative phosphorylation state could be modified to quantify any protein.

Because these ELISA's are performed in 96-well plates, multiple samples can be analyzed simultaneously. Compared to using immunoprecipitations to study phosphorylation, much less sample is required. Also, because less protein is needed and/or less time is required due to analysis of multiple samples, this procedure may be more cost-effective than immunoprecipitation or 2-D electrophoresis and immunoblotting techniques when attempting to quantify changes in StAR phosphorylation.

Chapter 4

Role of Protein Kinase A (PKA) in Steroidogenesis by Large and Small Luteal Cells

Introduction

One of the most intriguing questions regarding progesterone production by the corpus luteum (CL) is why large luteal cells are constitutively steroidogenic while small luteal cells have lower basal production of progesterone that increases in response to hormonal stimulation (55)? Surprisingly, not many studies have tried to address this question. The transport of cholesterol to the mitochondria and across the mitochondrial membrane is the rate-limiting step in steroidogenesis and is the step most acutely influenced by second messengers (reviewed in 193). Factors that activate protein kinase A (PKA) increase progesterone production, while activation of protein kinase C (PKC) inhibits (53). Therefore, factors that confer a constitutively steroidogenic phenotype most likely influence the transport of cholesterol across the mitochondrial membrane and may be regulated differently by PKA in the two steroidogenic cell types of the CL.

Cyclic AMP (cAMP) stimulates progesterone secretion by small but not large ovine luteal cells (256). This indicates that steroidogenesis by large cells is independent of the cAMP/PKA pathway, substrates of the cAMP/PKA pathway have been modified, or

PKA is already active in large cells. The latter possibility seems most likely because treatment with a specific PKA inhibitor caused a decrease in basal steroidogenesis by large luteal cells of sheep (97). Therefore, it appears that steroidogenesis in large cells involves PKA. However, the fact that cAMP does not stimulate secretion of progesterone from large cells indicates that PKA may be tonically active in this cell type. While the possibility of tonically active PKA in large cells is interesting and is not a new theory (8, 14, 243), information is limited. Therefore, the aim of the current study was to investigate the possibility that large luteal cells are constitutively steroidogenic because they have tonically active PKA. Steroidogenic acute regulatory protein (StAR) is responsible for the transport of cholesterol to the mitochondria and across the mitochondrial membrane, and StAR is regulated by the cAMP/PKA pathway through transcriptional and post-translational events (reviewed in 193). Therefore, the expression and phosphorylation state of StAR was quantified in the two steroidogenic cell types as a biochemical marker of PKA activity.

Materials and Methods

Materials

Tissue culture supplies (plates, pipettes, etc.) were purchased from Sarstedt Inc., Dulbecco's Modified Eagles Medium (DMEM) and antibiotic/antimycotic concentrate (containing penicillin G, streptomycin, and amphotericin B) were from Mediatech Inc., and fetal bovine serum (FBS) was from Gemini Bio-Products. Radioiodinated cyclic AMP (cAMP) was purchased from Perkin Elmer Life Sciences. Chemicals for cell

treatments included: myristoylated protein kinase inhibitor (PKI) 14-22 amide from BioSource Inc., forskolin and high-density lipoprotein (HDL) isolated from human plasma were purchased from Calbiochem/Novabiochem Corporation.

Animals

All experimental protocols involving the use of animals were approved by the university Animal Care and Use Committee. Western range ewes were superovulated during non-breeding and breeding seasons to provide corpora lutea for large and small luteal cell purifications. During the non-breeding season, ewes were prepared for superovulation by application of an intra-vaginal progestin releasing sponge (J&M Veterinary Services) for 12-14 days (ewes in the winter breeding season did not receive progestin). Following removal of the sponges, 1500 I.U. of equine chorionic gonadotropin (eCG) were injected to stimulate follicular growth, and 100μg of gonadotropin releasing hormone (GnRH) given 48 hours post-eCG to induce multiple ovulations. Ten days after GnRH treatment, ewes were anesthetized with pentobarbital (5mg/lb body weight), and corpora lutea collected surgically by mid-ventral laparotomy. Corpora lutea were pooled from 2-3 ewes per day, luteal tissue enzymatically dissociated into single cells, and preparations of small and large steroidogenic luteal cells separated by centrifugal elutriation (16). This procedure was repeated on five separate days (n = 5).

Cell Culture

Small (SLC) steroidogenic cells were plated at 2-3 × 10^6 per 60mm tissue culture plate, and 5-6 × 10^5 or 2.5-3 × 10^5 large (LLC) steroidogenic cells were plated per 60mm or 6-

well tissue culture plate respectively. Both cell types were plated overnight in culture medium (DMEM plus 10% heat-inactivated FBS, 100 I.U./ml penicillin G, 100μg/ml streptomycin, and 250ng/ml amphotericin B). Cells were cultured in an incubator at 37° C, in 5% CO_2.

Treatments

The PKA inhibitor used for these studies was derived from amino acids 14-22 of the skeletal muscle cAMP-dependent protein kinase inhibitor and was myristoylated at its N-terminus to enhance cell permeability. The PKI solution was prepared fresh each day by diluting lypholized PKI in serum-free DMEM to a final concentration of 500μM. Forskolin, which activates adenylyl cyclase and ultimately the cAMP/PKA pathway (257), was prepared in dimethyl sulfoxide (DMSO) at a concentration of 25mM. Cells were treated in a 2 × 2 factorial resulting in four groups for each cell type: 1.) non-treated containing 0.04% DMSO, 2.) 10μM forskolin, 3.) 50μM PKI with 0.04% DMSO, and 4.) 10μM forskolin with 50μM PKI. Treatments were added to duplicate plates. The day after elutriation, media were aspirated and replaced with serum-free culture medium. Six hours later, fresh serum-free culture media containing 50μM PKI for the appropriate groups were added, and cells incubated for one hour. Following the PKI loading, media were removed and replaced with fresh serum-free culture medium containing the appropriate treatments, and all groups received 50μg/ml human HDL to supply cholesterol. Cells were incubated 4 hours and then media were collected and cells washed twice with Tris-buffered saline (TBS). Media and cells were frozen at -20°C until analysis.

Progesterone Radioimmunoassay

Concentrations of progesterone in media were determined by radioimmunoassay (RIA) (248). The inter-assay coefficient of variation (CV) was 15.6, 3.4, and 7.6% for the low, medium, and high concentration quality controls respectively. The intra-assay CV averaged 8.9 and 17.8% at 20 and 80% respectively on the theoretical standard curve for three assays. Sensitivity of the assays averaged 980 femtograms per tube.

cAMP RIA

Concentrations of cAMP in media were determined by radioimmunoassay (258). Standards and samples were acetylated to increase sensitivity of the assay (259, 260). Acetylation of standards and samples was accomplished by adding 10µl of acetylating reagent (1 part acetic anhydride, 2 parts triethylamine) per 200µl of standard or unknown followed by vortexing for 2 seconds. The acetylating reagent was prepared fresh and used within 7 min. The assay had an average intra-assay CV of 7.9 and 15.7% at 20 and 80% on the theoretical standard curve respectively, and an inter-assay CV of 6.9 and 4.6% respectively for low and high concentration quality controls. The average sensitivity was 92 femtograms per tube.

Preparation of Cell Lysates and Sandwich ELISA's

Cells were thawed and collected in 200-300µl of cold homogenization buffer [250mM sucrose, 10mM Tris-HCL, pH 7.4, 10mM EDTA, 1mM sodium orthovanadate, protease inhibitor cocktail (Roche Diagnostics Inc.) and phosphatase inhibitor cocktail 1 (Sigma-

Aldrich) added at manufacturer recommended concentrations] per plate. Cells from duplicate plates were pooled and lysed by sonication with a Branson Sonifier using 4 × 10 second bursts on ice. Lysates were boiled at 100°C for 4 min immediately following sonication, and concentrated in a Savant Speed Vac for 20-40 min. Total protein concentration was determined by protein assay (Pierce Biotechnology) using bovine serum albumin (BSA) as the standard.

The concentration and relative phosphorylation state of StAR in each sample was determined using the modified sandwich ELISA's described in chapter 3.

Statistical Analyses

Data for concentrations of progesterone and cAMP in media, concentration of StAR protein, and percentage of StAR phosphorylation were analyzed using analysis of variance in the statistical analysis system (SAS). Potential outliers were excluded from analysis if their value was outside a 95% confidence interval calculated from the remaining values. Only 1 out of 40 values for concentrations of cAMP in media was excluded, and 4 out of 40 for relative StAR phosphorylation. Duncan's multiple range test was used to compare means from all four responses measured within each cell type and across treatments. Least significant differences were used to compare means for relative percentage of endogenous StAR phosphorylation between large and small luteal cells under non-treated conditions. A correlation analysis among the four responses measured in the experiment was performed using the average value for each response, and data from large and small luteal cells were pooled.

Results

Treatment of small luteal cells with forskolin caused an approximately 7.8-fold increase ($p < .05$) in concentrations of progesterone in media (Figure 4.1 A). Small cells treated with PKI alone had a 68% decrease in basal progesterone synthesis ($p = .58$ compared to non-treated). Co-treating PKI with forskolin inhibited ($p < .05$) the forskolin-induced increase in progesterone secretion to 1.1 fold of the non-treated control. Basal concentrations of cAMP in media were similar between non-treated small luteal cells and those receiving PKI alone ($p = .99$). Forskolin caused an approximately 5-fold increase in concentrations of cAMP in media ($p < .05$), and co-treatment with PKI did not inhibit the forskolin-induced increase in cAMP.

Concentrations of progesterone in media increased 2.4 fold (Figure 4.1 B) following forskolin treatment of large cells. Co-treating PKI with untreated or forskolin-treated large cells caused significant ($p < .05$) decreases in basal progesterone secretion (~84 and 77% decreases respectively compared to untreated). Basal secretion of cAMP into media was similar between non-treated and PKI-treated cells ($p = .80$). There was an approximately 5.7-fold increase ($p < .05$) in concentrations of cAMP in media following forskolin treatment. Forskolin and PKI co-treatment caused an approximately 2.2 fold increase in concentrations of cAMP in media, although this was not significantly higher than non-treated cells.

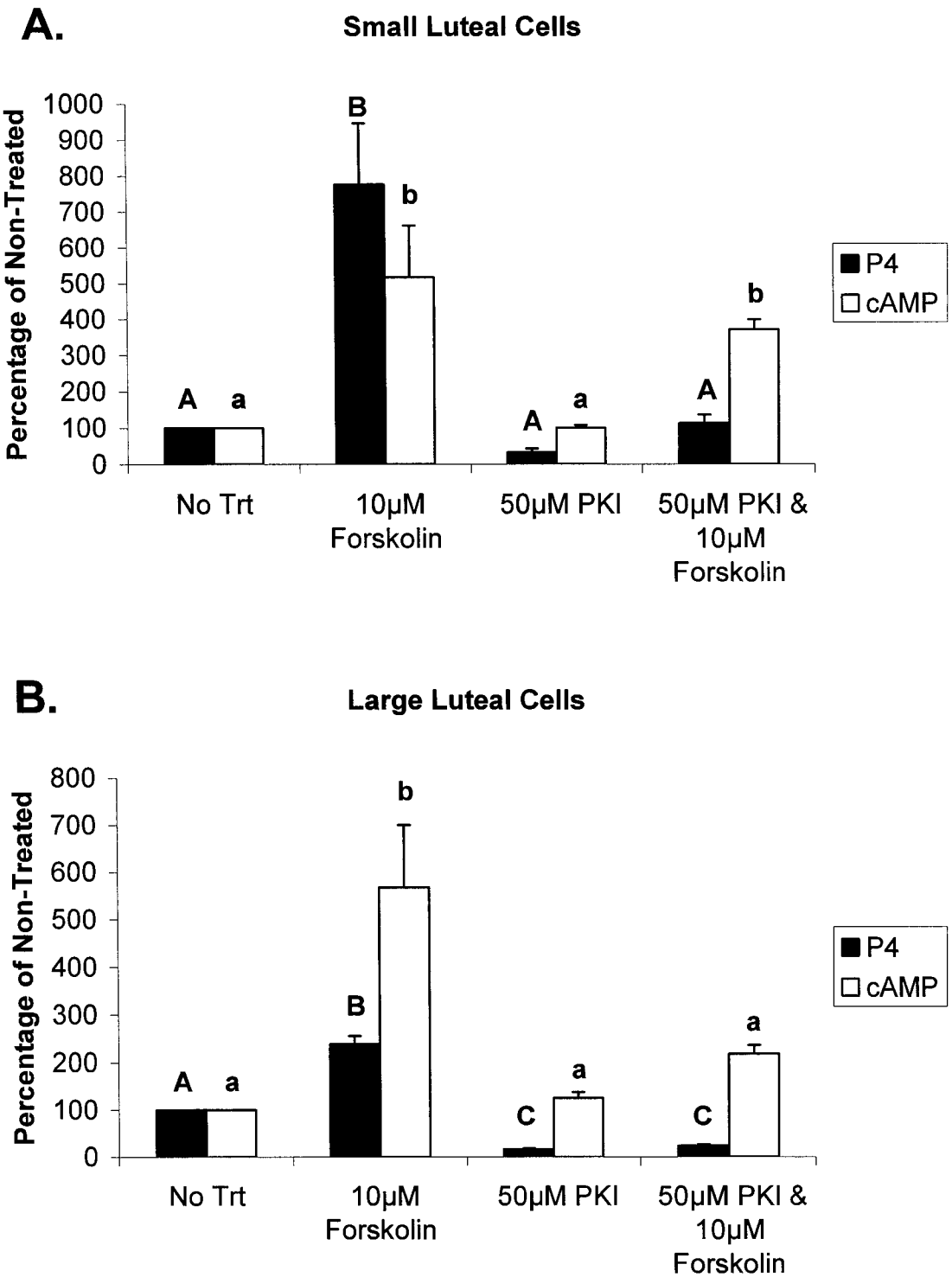


Figure 4.1: Concentrations of progesterone (P4) and cAMP in media following culture of small (graph A) and large (graph B) luteal cells. Error bars indicate one SEM; columns with different letters of the same case are significantly different ($p < .05$).

There were no significant differences in concentrations of StAR protein between treatment groups in either cell type (Figure 4.2). Ten luteal cell samples, consisting of both cell types and all treatments, were re-analyzed for concentration of StAR protein after being diluted for quantification of relative StAR phosphorylation. The 10 samples averaged 19.7 ± 2.9ng of StAR protein per well, with a targeted amount of 20ng per well.

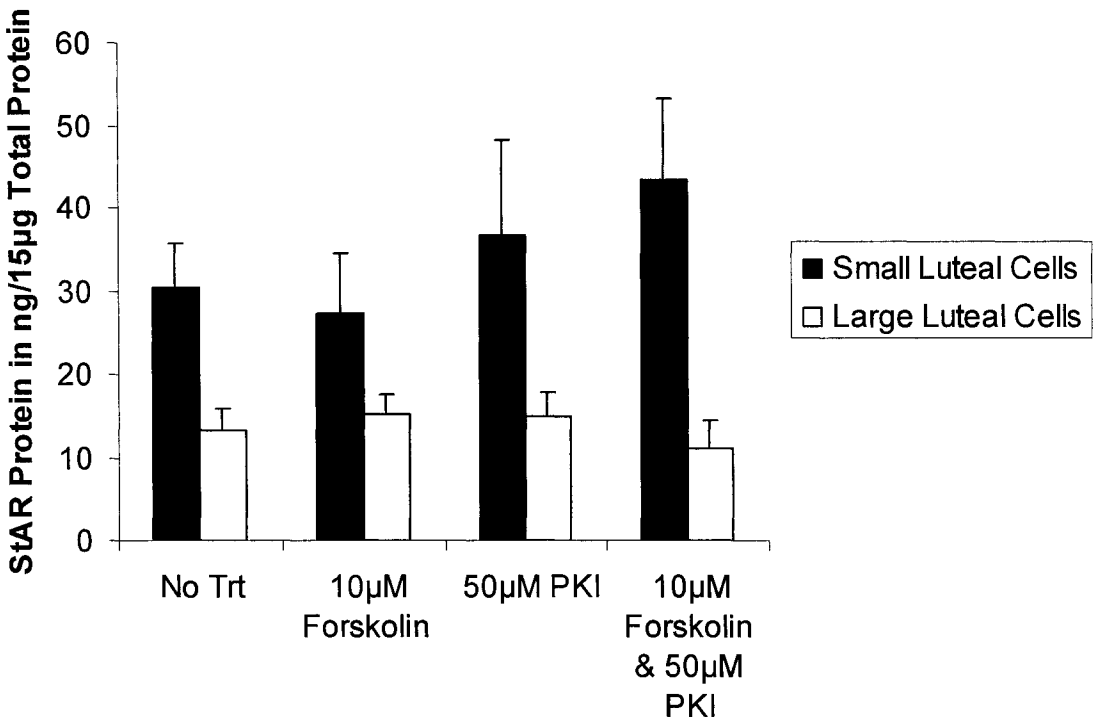
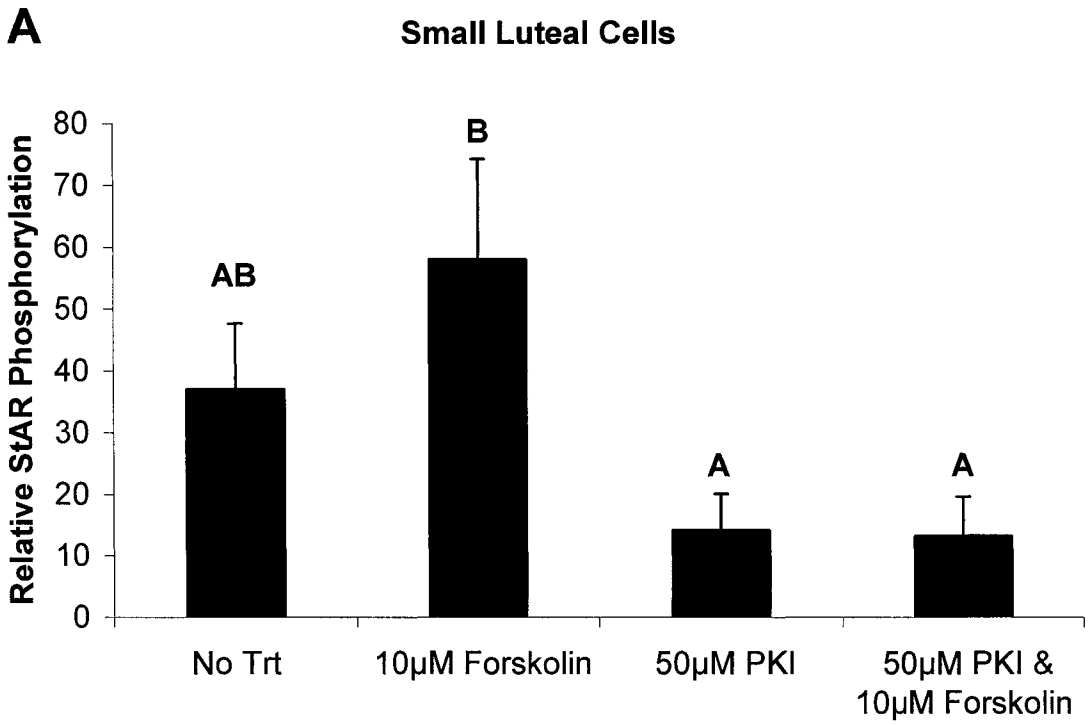


Figure 4.2: Concentrations of StAR protein in preparations of small and large luteal cells expressed as ng of StAR per 15µg total protein. Error bars indicate one SEM; there were no significant differences between treatments in either cell type.

Small cells treated with PKI or PKI and forskolin had significantly ($p < .05$) less relative StAR phosphorylation than forskolin-treated cells (Figure 4.3 A). In large cells (Figure 4.3 B), there was no increase in StAR phosphorylation following forskolin treatment.

Treatment with PKI in the presence or absence of forskolin resulted in a significant ($p < .05$) decrease in StAR phosphorylation compared to non-treated or forskolin-treated cells. Comparing the phosphorylation state of StAR between small and large cells revealed that the phosphorylation state of StAR is significantly higher in large than small cells ($p < .05$) under basal conditions (Figure 4.4).



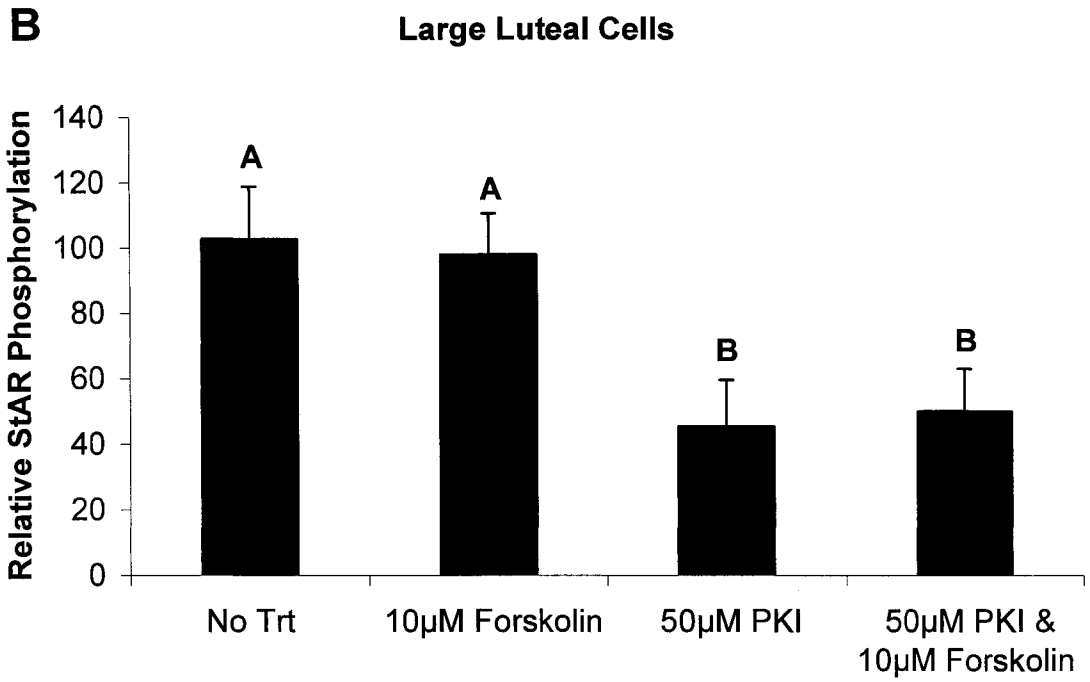


Figure 4.3: Relative StAR phosphorylation in small (graph A) and large (graph B) luteal cells, expressed as percentage of StAR phosphorylation compared to forskolin/PMA-treated mixed luteal cells that were used as the 100% phosphorylated standard. Error bars indicate one SEM; columns without common letters are significantly different ($p < .05$).

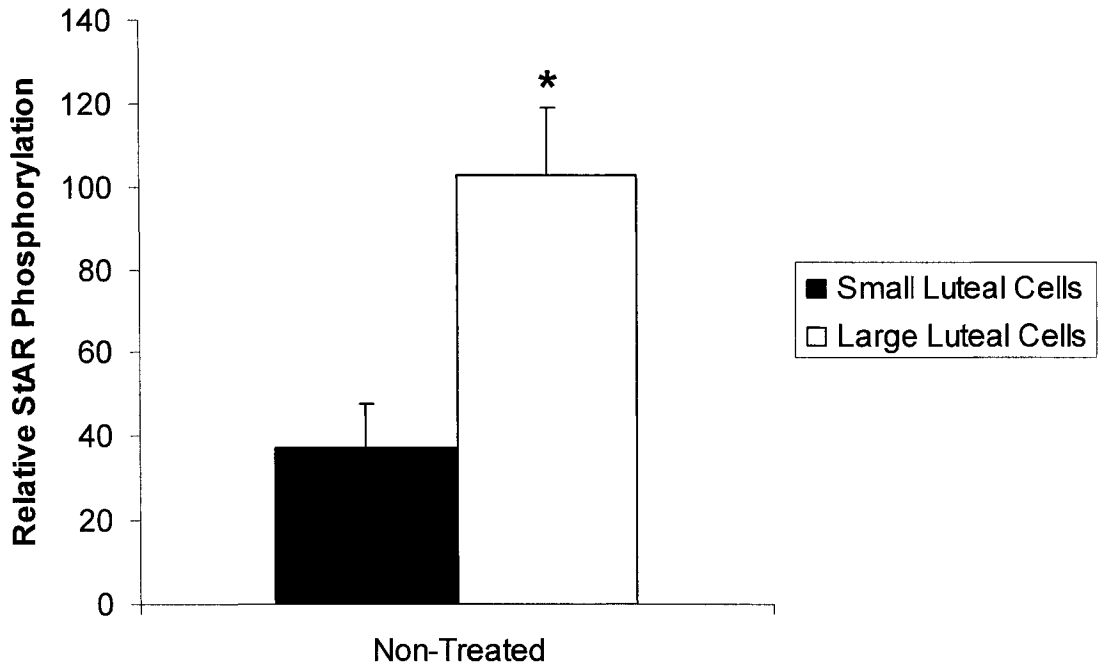


Figure 4.4: Comparison of endogenous StAR phosphorylation under basal conditions between large and small luteal cells, expressed as percentage of StAR phosphorylation relative to forskolin/PMA-treated mixed luteal cells. Asterisk denotes significant difference ($p < .05$).

Correlation analysis was performed between the four responses analyzed in this experiment using data from both large and small luteal cells (1. concentrations of progesterone in media, 2. concentrations of cAMP in media, 3. concentration of StAR protein, and 4. relative StAR phosphorylation) (Table 4.1). Additionally, correlation between responses was plotted for small and large luteal cells individually (data not shown) to verify that average correlations were representative of each cell type. As expected, concentrations of cAMP and progesterone in media were significantly correlated at $r = .78$ ($p < .05$). Relative StAR phosphorylation and progesterone concentrations in media were also significantly correlated ($r = .71$, $p < .05$). There was no correlation between concentrations of StAR protein and progesterone ($r = -.29$, $p = .49$).

	Progesterone in Media	Relative StAR Phosphorylation	StAR Protein Concentration	cAMP in Media
Progesterone in Media	r = 1.0	r = .71 p = .05	r = -.29 p = .49	r = .78 p = .02
Relative StAR Phosphorylation	r = .71 p = .05	r = 1.0	r = -.76 p = .03	r = .17 p = .68
StAR Protein Concentration	r = -.29 p = .49	r = -.76 p = .03	r = 1.0	r = .16 p = .71
cAMP in Media	r = .78 p = .02	r = .17 p = .68	r = .16 p = .71	r = 1.0

Table 4.1: Correlation analysis between the four responses analyzed. Data represent the mean of five replicates for each response, and data from small and large cells were pooled.

Discussion

Ligand binding to the LH receptor stimulates steroidogenesis in small luteal cells by activating PKA, and this effect can be mimicked with pharmacological activators of PKA (16); thus progesterone production by small cells served as a control for determining the level of PKA inhibition induced by PKI treatment. The increase in secretion of progesterone caused by forskolin treatment of small cells was eliminated by co-treatment with PKI, indicating that the concentration of PKI used inhibited almost all PKA-dependent steroidogenesis. Interestingly, small cells had an approximately 68% decrease in basal progesterone synthesis following PKA inhibition. In large cells, the decrease in basal steroidogenesis following PKA inhibition was greater than in small cells (~84% decrease, $p < .05$ different from non-treated), and more than reported in another study (~50-60%) (97). This might be expected as the dose of PKI used in the present report was twice that used by Diaz *et al.* (2002). Therefore, it appears that nearly all the progesterone produced by large cells under basal conditions is due to PKA-induced events. The

increase in secretion of progesterone by large cells following forskolin treatment was likely due to small cell contamination. Purified large luteal cells may contain approximately 20% small luteal cells existing in clumps or attached to large cells, while small steroidogenic cell preparations have no significant large cell contamination (16). Analysis of concentrations of progesterone in media provides evidence that basal progesterone synthesis by both cell types is dependent on PKA with the key difference that small cells have a greater capacity to respond to PKA activation with enhanced steroidogenesis. It appears likely that the reason large cells do not respond to LH/hCG or pharmacologic activation of PKA with enhanced steroidogenesis is because PKA is fully active.

In the one previous study where PKI was used to inhibit PKA in small and large steroidogenic luteal cells, there was no control for possible toxic effects of PKI (97). Presumably, PKA is important for cellular functions other than steroidogenesis, so blocking PKA activity could decrease progesterone synthesis simply by causing cellular toxicity. Concentrations of cAMP in media were analyzed to test for possible toxicity due to the PKI treatment. If cAMP production is unaffected by PKI treatment, than at least in regards to the steroidogenic pathway, it can be assumed that everything up-stream of PKA activation is normal, and any effect of PKI is due to inhibition of PKA. Concentrations of cAMP in media were measured in this study because they are correlated with intracellular concentrations of cAMP in ovine luteal cells (256). Once again, small cells served as the control. There was a significant increase in cAMP secretion by small cells following forskolin treatment. Small cells treated with PKI and

forskolin had significantly higher concentrations of cAMP in media than non-treated cells, and were not significantly different from forskolin-treated cells. Thus, there is no evidence of PKI-induced toxicity in small cells. In large cells, forskolin caused a significant increase in cAMP secretion. Basal concentrations of cAMP were similar between non-treated and PKI-treated large cells. There was an approximately 2.2-fold increase in cAMP production with PKI and forskolin co-treatment compared to non-treated cells. While this was not a statistically significant increase, if the forskolin-treated cells are excluded from statistical analysis, this becomes a significant ($p < .01$) increase over non-treated cells. Even though PKI and forskolin co-treatment caused significantly lower concentrations of cAMP in media than forskolin alone, this is probably not sufficient to explain the nearly complete inhibition of basal progesterone secretion by PKI.

There were no significant differences between treatments in either cell type in concentrations of StAR protein on a per microgram of protein basis. Possibly, treating cells in serum free media may have affected protein expression, or the treatment time (4 hours) may have been too short to induce changes in StAR expression. However, there was no correlation between concentrations of StAR protein and progesterone secretion. Therefore, even under different experimental conditions, it appears unlikely that a change in StAR expression is a primary mechanism behind PKA-induced steroidogenesis in luteal cells.

In small cells, PKI caused a significant inhibition of StAR phosphorylation compared to forskolin alone, indicating that PKI effectively blocked any PKA-induced phosphorylation of StAR. In large cells, basal phosphorylation of StAR was high and did not increase with forskolin treatment. Furthermore, there was a significant decrease in the relative percentage of StAR phosphorylation under basal or forskolin-stimulated conditions when PKA activity was inhibited in large cells. The StAR protein is phosphorylated significantly more in large compared to small cells under basal conditions. The higher basal phosphorylation of StAR induced by PKA could explain why large cells produce large amounts of progesterone constitutively.

As expected, there was a significant correlation between concentrations of cAMP and progesterone in media. Interestingly, a significant correlation was found between the percentage of StAR phosphorylation and progesterone synthesis. This is the first evidence that StAR phosphorylation by PKA stimulates progesterone synthesis in large cells, in spite of the fact that this cell type does not respond to pharmacologic activation of PKA with enhanced steroidogenesis (16, 53). Because there was no significant correlation between concentrations of StAR protein and progesterone secretion, this indicates that phosphorylation of StAR and not StAR expression is the key event in PKA-induced steroidogenesis. It is important to note that activators and inhibitors of PKA were used in this experiment; therefore the significant correlation between StAR phosphorylation and concentrations of progesterone in media is most likely due to changes in phosphorylation that occurred at PKA motifs. StAR has potential PKC phosphorylation sites (55), and because PKC down-regulates steroidogenesis in luteal cells (53), it is possible that

changes in StAR phosphorylation induced by PKC would be negatively correlated with progesterone synthesis.

In conclusion, these data provide strong evidence that the reason large luteal cells are constitutively steroidogenic is because they have tonically active PKA. Inhibiting PKA causes a substantial decrease in basal progesterone secretion by large cells, which is accompanied by a significant decrease in the relative phosphorylation state of StAR. Furthermore, phosphorylation of StAR, but not changes in StAR quantity, is the primary effect of PKA to regulate StAR activity in both steroidogenic cell types. Analysis of StAR phosphorylation provides data that directly link PKA activity and steroidogenesis in large cells, as well as providing further evidence that PKA is constitutively active in large cells.

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