AN ABSTRACT OF THE THESIS OF

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Title: Nongenomic Inhibition of Oxytocin Binding by Progesterone in Ovine Uteri.

Abstract Approved:

Fredrick Stormshak

Effects of progesterone (P_4) and mifepristone (RU 486) on binding of oxytocin (OT) to its ovine uterine receptor (OTR) were studied in two experiments. Two additional experiments were conducted to evaluate specific binding sites for P_4 in endometrial plasma membranes. In Exp. 1, ovariectomized ewes were injected with estradiol-17β (E_2) and P_4 to simulate an abbreviated estrous cycle (7 days) and then treated daily with 25 μg E_2 plus the following steroids for an additional 3 days: Grp. 1) controls (none), 2) 10 mg P_4; 3) 10 mg P_4 + 10 mg RU 486. Endometrium removed on Day 11 was stored at -80°C until analyzed for OTR. For Exp. 2, endometrial plasma membranes (1 mg protein/ml) from control ewes were incubated for 1 h (25°C) with the following steroids: 1) controls (C), 2) P_4 (5 ng/ml) and 3) P_4 + RU 486 (10 ng/ml), and analyzed for OTR. Scatchard analysis revealed high affinity OT binding sites (K_d of 1.01 X 10^{-10}M). *In vivo* and *in vitro* treatment with P_4 suppressed the

binding of OT to OTR ($p<0.01$) and treatment with RU 486 blocked the effect of P_4 ($p>0.05$). In Exp. 3, membranes recovered from control ewes as in Exp. 1 were analyzed for specificity of P_4 binding using [^{3}H] P_4 and [^{3}H] promegestone (R5020). Scatchard analysis revealed high affinity P_4 and R5020 binding sites (K_d 1.2 x 10^{-9} and 1.74 x 10^{-10}M, respectively). Binding of [^{3}H] R5020 to plasma membranes was inhibited by competition with a 200-fold excess of unlabeled R5020, P_4, RU 486 and OT (37-49%) but not by E_2, cortisol, or testosterone. In Exp. 4, membranes were incubated *in vitro* with P_4 or P_4 + RU 486 and then analyzed for specific binding of [^{3}H] R5020. Progestin binding was greater in membranes exposed to P_4 compared to controls or those incubated with P_4 + RU 486 ($p<0.05$). These data suggest a direct interaction between P_4 and OTR at the level of the membrane that suppresses binding of OT to the OTR.

Nongenomic Inhibition of Oxytocin Binding by Progesterone in Ovine Uteri

by

Kathrin Anson Dunlap

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In honor of Jenny, Maggie, Mom and Dad.

NONGENOMIC INHIBITION OF OXYTOCIN BINDING BY PROGESTERONE IN OVINE UTERI

REVIEW OF THE LITERATURE

CHARACTERISTICS OF THE ESTROUS CYCLE

The uterus is greatly influenced by the inherent cycle of hormones to which it is exposed in the adult female. The estrous cycle is divided into four main periods: proestrus, estrus, metestrus, and diestrus. At each of these periods, there are differing hormonal profiles that cause behavioral differences, as well as physiological changes in the reproductive tract environment. An ovine estrous cycle is on average 17 days in duration but can range from 13 to 19 days (Stabenfeldt et al., 1969c; Bazer et al., 1998; Senger, 1999).

Estrus

The period of estrus is primarily defined by behavior. During standing estrus the female is most receptive to mating, and copulation is most likely to occur. The duration of estrus varies among species. In the ewe, this period has a range of 18 to 48 hours, but on average lasts 30 hours (Bazer et al., 1998; Senger, 1999). The beginning of the estrous cycle is usually defined by the onset of behavioral estrus.

Estrus is also defined by the characteristically high levels of systemic estrogen. At the onset of estrus estrogen levels are at their greatest concentrations, which results in a dramatic up-regulation of uterine estrogen and progesterone receptors (Vallet et al., 1990; Wathes and Hamon, 1993).

The release of follicle stimulating hormone (FSH) allows for the recruitment of follicles for ovulation (Stabenfeldt et al., 1969). The preovulatory or ovulatory follicle increases its production of estrogen as it develops. The follicular estrogen production results in maximal levels of estrogen receptor expression, as well as an increased feedback upon the anterior pituitary (Bazer et al., 1998). The pre-ovulatory luteinizing hormone (LH) surge from the anterior pituitary coincides with increased

blood flow to the ovary, increased prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and prostaglandin E_2 (PGE_2) production by the ovary, as well as increased collagenase synthesis. These factors result in ovulation. Ovulation typically follows the onset of behavioral estrus by 24 to 30 hours in the ewe (Bazer et al., 1998; Senger, 1999). Ovulation marks the boundary between the follicular and luteal phases of the estrous cycle. The luteal phase of the cycle begins with the period of metestrus.

Metestrus

Metestrus is the brief period following estrus in which both estrogen and progesterone levels are relatively low. During this time the ovarian structure known as the corpus luteum is formed as a result of the process of luteinization (Bazer et al., 1998; Senger, 1999). Luteinization is characterized by the transition of the follicular theca interna and granulosa cells into small and large luteal cells, respectively (Corner, 1919; Fortune and Hansel, 1979; Milvae et al., 1991). This development occurs concomitantly with a shift from estrogen to progesterone production induced by the ovulatory surge of LH. The LH surge also

plays a role in luteal oxytocin secretion. The large luteal cells (derived from the granulosa cells) possess oxytocin contained in secretory granules that form in the Golgi apparatus and upon appropriate stimulation are transported to the plasma membrane and are secreted (Fields and Fields, 1996). In studies conducted using cultured bovine granulosa cells, Voss and Fortune (1991) demonstrated that cells collected prior to the LH surge secreted less oxytocin *in vitro* than cells collected after the LH surge.

Luteal cells are steroidogenic and are thus responsible for progesterone production. The corpus luteum is the dominant source of progesterone during the estrous cycle. This endocrine gland begins secreting progesterone as early as the metestrous phase of the cycle but does not attain its maximal size and function until between days 7 and 14 of the ovine cycle (diestrus) (Bazer et al., 1998; Senger, 1999).

Diestrus

Diestrus is characterized by the dominant levels of systemic progesterone produced by the corpus luteum. As previously stated, the

levels of progesterone produced by the corpus luteum do vary throughout diestrus. Data collected by Stormshak et al. (1963) lend credence to the hypothesis that the quantity of progesterone released into the blood is positively correlated to luteal progesterone content. Average weight and total progesterone content of the corpus luteum are maximal on day 10 of the cycle and remain constant until the time of regression (Stormshak et al., 1963). Supporting data collected by Plotka and Erb (1967) demonstrate that progesterone levels in the cycling ewe increase following metestrus to their maximum point during diestrus (9 to 11 days postestrus) and then decrease again 13-15 days after estrus; i.e., during proestrus. Stabenfeldt et al. (1969c) reported that plasma concentrations of progesterone gradually increased to day 10 of the ovine cycle and were maintained until day 16. Koligian and Stormshak (1977) also detected increasing serum concentrations of progesterone to day 10 of the estrous cycle. The variation in progesterone levels is due to changes in the number of small and large luteal cells. Luteinizing hormone is the major luteotropin in the cow and ewe (Simmons and Hansel, 1964; Karsh et al., 1970). Increased binding of LH to receptors on luteal cells is positively correlated with progesterone levels and luteal weight (Diekman et al., 1978a,b; Jones et al., 1992). The regulatory

mechanisms regarding progesterone production by large luteal cells are not as yet well defined.

Proestrus

Reduction in progesterone synthesis and secretion is essential as a prelude to the onset of estrus. Within the cow, ewe, and gilt, plasma progesterone levels decline to less than 1 ng/ml during the proestrous period (Stabenfeldt et al., 1969a,b,c). The decreased progesterone production allows for an increase in gonadotropin-releasing hormone (GnRH) production in the hypothalamus because progesterone feeds back on the hypothalamus in a negative manner to block the release of GnRH during the estrous cycle (Nett et al., 1974). Gonadotropin-releasing hormone causes the release of FSH and LH from within the anterior pituitary (Adams et al., 1979).

At proestrus (day 14-17 in the ovine), estrogen from maturing ovarian follicles further stimulates increases in endometrial concentrations of estrogen and oxytocin receptors, thus ensuring that oxytocin from the posterior pituitary and subsequently the corpus luteum

stimulates the pulsatile releases of $PGF_{2\alpha}$ that ensure luteal regression. A frequency of 5 pulses of $PGF_{2\alpha}$ within a 24 hour period are necessary to initiate luteal regression (McCracken et al., 1973).

Adequate progesterone exposure during the early to mid-luteal phase of the estrous cycle is also important for initiation of the ovarian and uterine mechanisms that lead to luteolysis in the ewe. Morgan et al. (1993) demonstrated that the uterine endometrium must experience progesterone stimulation for a finite period of time followed by a down-regulation of progesterone receptors prior to luteolysis. This supports the 8-10 day progesterone priming schedule established by McCracken et al. (1984). Using ovariectomized ewes receiving 12 days of progesterone treatment Vallet et al. (1990) observed an increase in endometrial oxytocin receptor concentration and $PGF_{2\alpha}$ responsivness to an oxytocin challenge.

At the time of luteolysis, oxytocin binding to its receptor stimulates a double positive feedback loop which causes the release of $PGF_{2\alpha}$ from the uterine endometrium while stimulating the oxytocin receptor phospholipase C system (Mirando et al., 1993; Ivell and Walther, 1999; Ivell et al., 2000). At the time of luteal regression, uterine oxytocin receptor concentration is at its maximum (Vallet et al., 1990; Mirando et

al., 1993). Sheldrick and Flint (1985) hypothesized that luteal secretion of oxytocin may provide a means of amplifying the luteolytic-signal, thus ensuring luteal regression.

The $PGF_{2\alpha}$ pulses in sheep and cattle reach the corpus luteum via a countercurrent exchange mechanism (Mapletoft and Ginther, 1975; Mapletoft et al., 1976) Prostaglandin $F_{2\alpha}$ produced by the endometrium enters the uterine vein and then crosses through the vein wall enroute to the ovarian artery (Dobrowolski and Hafez, 1970; Ginther et al., 1973) Upon reaching the corpus luteum, $PGF_{2\alpha}$ acts as a luteolysin and further stimulates ovarian oxytocin secretion (Flint and Sheldrick, 1982).

As previously described, uterine $PGF_{2\alpha}$ and luteal oxytocin secretion occur as a consequence of a positive feedback mechansim, it has even been demonstrated that their pulsatile releases almost exactly coincide (Roberts et al., 1976; Flint and Sheldrick 1982; Flint et al., 1990; Mirando et al., 1993). It has also been demonstrated that during proestrus in the ewe, the degree of $PGF_{2\alpha}$ released is enhanced with low doses of oxytocin, with maximum levels occurring on the day of estrus (Roberts et al., 1976). In a study conducted by Flint et al. (1990) ovine luteal oxytocin secretion could be stimulated via administration of physiological doses of $PGF_{2\alpha}$ into the uterine vein or lymph vessel.

Additionally, Flint and Sheldrick (1982) demonstrated that intramuscular injection of the $PGF_{2\alpha}$ analogue, cloprostenol, resulted in a rapid release of oxytocin from the ovine ovary. Increased plasma oxytocin concentrations lasted up to 40 minutes. These observations were also apparent in *in vitro* studies. *In vitro* incubation of ovine endometrium results in spontaneous release of $PGF_{2\alpha}$ that can be enhanced by oxytocin in a dose dependent manner (Roberts et al, 1976). The demonstrated parallel systemic concentrations of oxytocin and $PGF_{2\alpha}$ throughout the estrous cycle suggest a fairly complex interrelationship (Flint and Sheldrick, 1983).

UTERO-OVARIAN FUNCTIONAL INTERRELATIONSHPS

The female reproductive tract exhibits a very highly developed source of internal interaction. The secretion of hormones, morphological structure of tract components, recognition and maintenance of pregnancy, and parturition are dependent upon complex relationships between the uterus and ovaries.

Uterine Classification

Classification of mammalian uteri is based upon the number of cervices possessed and the development of the uterine body and cornua (horns). Marsupials and rabbits are both classified as having duplex uteri, because they have two distinct cervices and vaginas (Senger, 1999). Many domestic livestock species, including the mare, goat, cow, and ewe, have a bipartite uterus. The bipartite uterus is comprised of a single cervix, primary uterine body, and two comparatively small uterine horns (Wiley et al., 1987). In contrast, the sow possesses a bicornuate uterus consisting of a small uterine body and very long horns. The length of the uterine horn varies among species and is determined at the stage of fetal development; the length is based on the extent of fusion between the paramesonphric or Mullerian ducts. For example, the mare exhibits a great deal of Mullerian duct fusion and thus has short uterine horns as compared to the limited Mullerian duct fusion that occurs in the fetal pig. The ewe possesses a uterus with intermediate length horns, with development that occurs between days 34 and 55 of fetal life (Wiley et al., 1987). The fourth classification of uterine types is characteristic of

primates and is referred to as simplex. The simplex uterus consists of a single chambered uterine body and no true horns (Senger, 1999).

Uterine Morphology

The uterus is a tubular organ comprised of three main tissue layers: serosa, muscularis, and mucosa/submucosa (Senger, 1999). These layers are also known as the perimetrium, myometrium, and endometrium. Each tissue layer plays a distinct role in pregnancy and parturition.

The perimetrium is, in essence, part of the peritoneum. It is the thin tissue layer that extends from the mesosalpinx to cover the uterine body. The perimetrium is the outermost protective layer of the uterine wall.

Beneath the perimetrium is the myometrium, which is a thick muscular layer. The myometrium is composed of a layer of circular smooth muscle surrounded by longitudinal smooth muscle. The stage of the reproductive cycle, and the resulting hormonal secretions that are characteristic of the particular stage, influence the "tone" of the

myometrium. During the period of estrus, the uterus receives an increased amount of estrogen exposure; consequently, the uterus is more turgid at that time than when it is under the influence of progesterone (Senger, 1999). Throughout gestation, progesterone is the dominant hormone in the uterine environment, which results in reduced binding of oxytocin to its receptor and allowing the myometrium to remain relaxed and conform to the changing shape of a conceptus (Ivell et al., 2000). At the time of parturition, the decreasing progesterone levels allow for the binding of oxytocin to its receptors, which are largely responsible for the induction of myometrial contractions. The contractile ability of the myometrium is required to expel the fetus and placental membranes upon stimulation by oxytocin.

The innermost layer of the uterus is the endometrium, which consists of both the mucosal and submucosal layers (Wiley et al., 1987; Senger, 1999). The mucosal layer of tissue is comprised of the luminal and glandular epithelium, which rests upon the submucosa that is primarily connective tissues (stroma; Arnold et al., 2001). Endometrial glands are required for normal uterine function and are responsible for the synthesis, secretion and transport of a complex array of proteins and related substances necessary for embryonic survival (Gray et al., 2001).

In humans and domestic livestock, endometrial adenogenesis is completed postnatally. Adenogenesis begins with the differentiation and budding of the glandular epithelium from the luminal epithelium, followed by invagination and extensive tubular coiling and concludes with branching morphogenesis throughout the uterine submucosa until reaching the myometrium (Wiley et al., 1987; Senger, 1999; Gray et al., 2001). Within the ovine, the extensive period of glandular development of the neonatal uterus occurs between days 9 and 26 (Wiley et al., 1987). These glands are not fully functional until they have been exposed to progesterone. The genesis of the ovine endometrial glands allows for the development of a dichotomous endometrium with the luminal surface possessing aglandular caruncular and glandular intercaruncular areas, both of which will remain into adulthood (Wiley et al., 1987).

In species that experience a menstrual cycle (i.e., primates), the endometrial layer is sloughed during menses. The growth and differentiation of the human endometrium has been evaluated *in vitro* and Arnold et al. (2001) have demonstrated that the stromal cells regulate the growth and development of epithelial cells. Stromal cells are also essential for the development of the uterine endometrium in estrual species such as the ewe. However, the endometrial layer of

estrual species is not destroyed, but rather undergoes a type of growth and regression that is dependent upon the hormones to which it is exposed to during the estrous cycle.

The physical appearance of the endometrium also varies among species. The endometrium is the site of embryo attachment or implantation and contains distinct structures that aid in this process. The endometrial layer of the mare and sow possesses longitudinal ridges or deep folds that allow for villous-type attachment while the cow and ewe possess caruncles (Wiley et al., 1987; Senger, 1999). Caruncles are vascularized, aglandular, button-shaped structures that are raised from the surface of the endometrium. These structures form the maternal site of placental attachment in the ruminant (Wiley et al., 1987; Senger, 1999).

Oxytocin Receptor Expression

The binding of oxytocin to its receptor is a crucial part of the reproductive process. Oxytocin receptors exist in varying concentrations in a variety of reproductive tissues (Sheldrick and Flint, 1985; Zhang et

al., 1992; Lamming et al., 1995; Wathes et al., 1996). Regulation of oxytocin receptor gene expression occurs at the level of transcription and is primarily influenced by the ovarian steroids: estrogen and progesterone (McCracken et al., 1984; Vallet et al., 1990; Morgan et al., 1993; Wathes and Hamon, 1993; Spencer et al., 1995; Bazer et al., 1998; Ivell and Walther, 1999).

The endometrium is a primary target tissue of oxytocin, and possesses twice the capacity of oxytocin receptors as compared to the myometrium (Roberts et al., 1976; Sheldrick and Flint., 1985; Vallet et al., 1990). There is evidence of high affinity oxytocin binding sites within both the endometrium and myometrium (Roberts et al., 1976; Sheldrick and Flint, 1985; Pliska et al., 1986; Vallet et al., 1990; Zhang et al., 1992; Wathes et al., 1996). Caruncular and intercaruncular endometrial tissue exhibit similar responses to steroid exposure, with the intercaruncular tissue maintaining a consistently greater concentration of oxytocin receptors (Zhang et al., 1992).

Increases in oxytocin receptor concentrations coincide with declining plasma concentrations of progesterone and the rise in ovarian venous levels of estradiol-17β (McCracken et al., 1984). Therefore, the initial increase in oxytocin receptors occurs in cyclic ewes when estrogen

receptors increase and progesterone receptors decrease in the endometrial epithelium. This change in estrogen and progesterone receptors results from a decrease in systemic progesterone levels and an increase in follicular estrogen production during late diestrus, proestrus, and estrus (McCracken et al., 1984; Wathes and Hamon, 1993; Spencer et al., 1995). At estrus, there is a peak number of oxytocin binding sites present in the endometrium (Roberts et al., 1976; Sheldrick and Flint, 1985; Lamming et al., 1995). As described in the previous section of this thesis, expression of oxytocin receptors on the luminal and superficial glandular epithelium appears to be responsible for the pulsatile release of $PGF_{2\alpha}$ necessary for luteolysis (Sheldrick and Flint, 1985; Bazer et al., 1998; Ivell and Walther, 1999).

In normally cycling ewes, progesterone inhibits endometrial oxytocin receptor synthesis for 10 to 12 days and by an autoregulatory mechanism eventually down-regulates its own receptor, especially in the endometrial luminal epithelium (Vallet et al., 1990; Wathes and Hamon, 1993; Wathes et al., 1996). The down-regulation of progesterone receptors precedes the up-regulation of oxytocin receptors in uterine cells (Vallet et al., 1990; Wathes and Hamon, 1993; Wathes et al., 1996). Zhang et al. (1992) demonstrated an increase in the concentration of

oxytocin receptors following a period of progesterone withdrawal in the ewe. The oxytocin receptor is initially expressed on luminal and superficial glandular epithelia on day 14 of the estrous cycle of the ewe, the caruncular stroma and deep glandular epithelium by cycle day 16, and is abundant in all endometrial cell types during estrus (Bazer et al., 1998). Oxytocin receptor concentrations continue to increase in cyclic ewes treated with progesterone for up to 30 days (Vallet et al., 1990; Vallet and Lamming, 1991). Conversely, chronic low dose exposure to estradiol decreases ovine endometrial oxytocin receptor concentrations during the estrous cycle (Wathes et al., 1996; Hazzard and Stormshak, 1997). In the absence of progesterone receptors, the endometrial epithelium responds to estrogen, expresses estrogen receptors, and up-regulates oxytocin receptor expression (Spencer et al., 1995). While the exact mechanisms by which these steroids function to regulate oxytocin receptors is yet unknown, it does appear that both progesterone and estrogen are ultimately responsible for the regulation of the oxytocin receptor. A more detailed discussion regarding the role of steroids in oxytocin receptor regulation will follow.

Ovariectomized ewes are frequently utilized to examine the effects of exogenous progesterone and/or estradiol on the concentration

of endometrial oxytocin receptors and plasma concentration of the metabolite 13,14-dihydro-15-keto prostaglandin $F_{2\alpha}$ (Vallet et al., 1990; Wathes et al., 1996). Using ovariectomized ewes it has been demonstrated conclusively that progesterone pretreatment increases both oxytocin receptor concentrations in endometrium and uterine responsiveness to oxytocin (Vallet et al., 1990; Wathes et al., 1996).

There is evidence for related peptides such as mesotocin and isotocin to bind to the ovine oxytocin receptor (Sheldrick and Flint, 1985; Pliska et al., 1986). There is also the possibility of multiple isoforms of the oxytocin receptor on the plasma membrane (Pliska et al., 1986).

The oxytocin receptor system is frequently studied *in vitro* using either myometrial cell cultures from rat or human uterus or endometrial epithelial cells from the ruminant uterus (Ivell et al., 1998). Sheldrick et al. (1993) evaluated oxytocin receptor binding in an ovine endometrial explant culture and found that estradiol had no stimulatory effect on oxytocin receptor synthesis by ovine endometrium. Oxytocin receptor binding activity was generally unrelated to the nature of the protein or hormonal supplement that was added to the culture medium (Sheldrick et al., 1993; Sheldrick and Flick-Smith, 1993). Consequently, any rise in receptor binding activity *in vitro* was determined not to relate to any

stimulation by medium supplements (Sheldrick et al., 1993). It was ultimately determined that an increase in oxytocin binding activity during culture required mRNA synthesis and therefore binding resulted from *de novo* synthesis of either the receptor itself or of a protein required for processing or activating the receptor (Sheldrick et al., 1993). The role of ovarian steroids in regulating oxytocin receptor mRNA levels will be discussed in the following section.

Utilizing bovine endometrium, ovarian follicular granulosa cells, and homologous transcription assays, Ivell et al. (1998) determined that while endometrial oxytocin receptors exhibited a cycle-dependent regulation *in vivo*, in culture there was a spontaneous up-regulation of the oxytocin receptors regardless of the time of tissue collection during the estrous cycle. Concentrations of bovine oxytocin receptor mRNA were also evaluated and shown to be at maximal levels prior to estrus; the point at which estradiol concentrations increased while progesterone was decreasing (Stewart et al., 1993; Ivell et al., 1998).

Cultured ovine endometrial epithelial cells spontaneously up-regulate the oxytocin receptor gene in the absence of progesterone (Sheldrick et al., 1993; Ivell and Walther, 1999). Endometrium of long-term ovariectomized and anestrous ewes express estrogen receptors

and oxytocin receptors without any additional stimulatory treatment (Bazer et al., 1998). In contrast, ovariectomized rats have demonstrated uterine oxytocin receptor up-regulation by estrogen and down-regulation by progesterone both *in vivo* and *in vitro* (Larcher et al., 1995).

The sow offers yet another example of uterine tissue responsiveness to oxytocin. The endometrial, myometrial, and stromal cells of the sow possess oxytocin receptors in differing concentrations (Soloff and Swartz, 1974; Hu et al., 2001). The uteri of both the sow and the mare are unique because they have been shown to transcribe the oxytocin-neurophysin I (OT-NP I) gene into mRNA (Trout et al., 1995; Behrendt-Adam et al., 1999). Thus, uteri of these two species synthesize and secret oxytocin that can act in an autocrine manner to regulate uterine function. The endometrial cells have a greater concentration of oxytocin mRNA than the glandular or stromal cells of the sow (Trout et al., 1995). However, luminal epithelial cells are less responsive to oxytocin exposure *in vitro* than the glandular or stromal cells of the sow (Hu et al., 2001). The luminal epithelial cells of the sow secrete oxytocin which promotes $PGF_{2\alpha}$ secretion into the uterine lumen (Hu et al., 2001). The role of uterine oxytocin secretion on regulation of reproductive function within mares is yet unknown (Behrendt-Adam et al., 1999).

Within the rat endometrial epithelium it appears that both oxytocin and oxytocin receptors are transported to apical compartments of the epithelial cell, suggesting an autocrine oxytocin system (Morel et al., 2001). The path for endometrial oxytocin secretion is vesicle-mediated exocytosis on the apical surface (Morel et al., 2001). Ultimately, the localization of the oxytocin receptor on the rat endometrial microvilli is the first example of a G-protein-coupled-receptor localized on the microvilli of uterine epithelium (Morel et al., 2001).

Changes in Oxytocin Receptor mRNA Levels

Oxytocin receptor mRNA expression within the pituitary gland remains constant throughout the estrous cycle while expression varies greatly within the endometrial and myometrial tissues (Feng et al., 2000). Concentrations of mRNA within the ovine endometrium have been shown to be very low during pregnancy and also low in the pregnant horn of a unilaterally pregnant ewe, suggesting trophoblast interferons also play a role in controlling the expression of the oxytocin receptor gene (Stewart et al., 1993). Oxytocin receptor gene expression is not

only tissue specific but also highly function related within the tissue (Feng et al., 2000).

The role of mRNA is important in the formation of different populations of oxytocin receptors. The detection of mRNA editing indicates that oxytocin receptor subtypes are present in the endometrium (Feng et al., 2000). It is proposed that alternative splicing of oxytocin receptor mRNAs correspond to oxytocin receptors in luminal epithelium and that unaltered oxytocin receptor mRNA may correspond to oxytocin receptors in caruncular stroma and deep glandular epithelium, which have been shown to respond to estrogen stimulation (Feng et al., 2000).

In a recent review completed by Ivell et al. (1998), these investigators concluded that the expression of the oxytocin receptor gene in the myometrium and endometrium is controlled by a complex regulatory system. Increased concentration of oxytocin binding sites detected at the onset of labor and at proestrus is due, at least in part, to an estrogen-induced up-regulation of oxytocin receptor gene expression (Larcher et al., 1995). Estrogen stimulation of oxytocin receptor gene expression is believed to play an important role in the stabilization of oxytocin receptor mRNA (Larcher et al., 1995).

Even with the lack of a functional estrogen response element, estrogen does play an important role in oxytocin and oxytocin receptor regulation. In the ovariectomized rat, the degree of oxytocin binding to its receptor was dramatically increased by a single injection of 0.5 μg of diethystilbestrol dipropionate (Soloff, 1975). The hypothesis of Soloff (1975) was that the increased sensitivity of the rat uterine samples was due to an increase in number and affinity of oxytocin receptors stimulated by the estrogen treatment. Evaluation of rat uterine oxytocin receptor mRNA levels throughout the estrous cycle demonstrated that the levels of oxytocin receptor mRNA and degree of oxytocin binding were positively correlated with the ratio of estrogen to progesterone (Larcher et al., 1995). The stimulatory effect of estrogen on oxytocin receptors has also been detected in the ewe, but not in the cow (Sheldrick and Flint, 1985; Vallet et al., 1990; Ivell et al., 2000). While the bovine does show a dramatic up-regulation in in the oxytocin receptor gene at the time of estrus, it has not been linked directly to estrogen exposure, because steroids lack a direct influence over oxytocin receptor gene constructs in DNA-protein binding and transfection assays (Ivell et al., 2000).

There is a close temporal relationship between oxytocin binding sites and the increase in oxytocin receptor mRNA. In the rat, oxytocin mRNA levels and oxytocin binding reach maximum levels concomitantly at proestrus; oxytocin binding sites undergo much more immediate changes than do the levels of oxytocin receptor mRNA (Larcher et al., 1995). At metestrus, oxytocin receptor mRNA levels were one-half the levels at proestrus but oxytocin binding was ten times lower than at proestrus (Larcher et al., 1995). During pregnancy a comparison of the recorded levels of oxytocin receptor mRNA to measured oxytocin binding produced a similar ratio as compared to the ratio of systemic estrogen and progesterone. The possibility that oxytocin receptor mRNA levels may not be perfectly correlated with oxytocin binding is supported by the observations of Larcher et al. (1995) who detected small variations in oxytocin receptor mRNA levels, but much larger variations in oxytocin binding. These findings support the possibility that a steroid hormone, such as progesterone, may exert an effect on oxytocin binding via a mechanism downstream of the oxytocin receptor mRNA, perhaps via a nongenomic mode of action (Larcher et al., 1995).

Chronic treatment of ewes with estradiol from the early to the late portion of the estrous cycle or administration of progesterone to ewes

during the late luteal phase of the cycle prevented oxytocin receptor gene transcription as determined by measuring changes in mRNA (Hazzard et al., 1998). The possibility that the interaction of steroids mediates oxytocin receptor regulation by gene suppression is supported by Larcher et al. (1995) who suggests that within the rat uterus progesterone acts to reduce uterine oxytocin binding via a protein synthesis-independent mechanism and does not involve a direct antagonism of the genomic action of estradiol. Evidence has been reported suggesting that the antagonistic effect of progesterone on oxytocin binding cannot be accounted for by its effect on oxytocin mRNA levels (Larcher et al., 1995). Collectively, it appears that the regulatory actions of steroids may be, in part, indirect and species specific.

Steroid Receptor Expression and Localization

The Uterus and Oviduct

There is evidence of progesterone receptors existing within the deep glands, caruncular stroma, deep stroma, myometrium, and

endometrium of the ovine (Vallet et al., 1990; Wathes et al., 1996). Estradiol has been shown to sustain progesterone receptors within the above mentioned tissues and stimulate progesterone receptors in epithelial cells (Vallet et al., 1990; Wathes et al., 1996). Conversely, progesterone will abolish progesterone receptors in epithelial cells over time and block estradiol's ability to sustain its own receptor (Koligian and Stormshak, 1977; Wathes et al., 1996).

Sequential progesterone treatment greatly reduces nuclear and cytoplasmic estrogen receptors and estrogen levels in ovaries, endometrium and the cervix of the female Macaque (West et al., 1986). In contrast to the regulation of the estrogen receptor, sequential progesterone treatment increases progesterone levels in excess of receptor levels in both the cytosolic and nuclear fractions of the ovary, oviduct, endometrium, and myometrium. After 2 weeks of progesterone treatment the only tissue showing an increase in number of occupied progesterone receptors was the endometrium. It appears that the stimulatory effects of progesterone may require higher levels of occupied nuclear progesterone receptors than do the suppressive effects of progesterone as evidenced by the expression of oviductal atrophy,

diminished cervical secretion, and myometrial quiescence (West et al., 1986).

In the guinea pig, hamster, and rat, uterine progesterone and estrogen receptors appear to be under the control of both estrogen and progesterone (Milgrom et al., 1973; Luu Thi et al., 1975; Leavitt et al., 1977; Vu Hai et al., 1977). *In vitro* studies have demonstrated that estrogen exerts a positive influence on receptors and requires both RNA and protein synthesis for upregulation (Milgrom et al., 1973; Leavitt et al., 1977; Vu Hai et al., 1977). Estradiol up-regulates expression of estrogen receptors within the uterine endometrium (Koligian and Stormshak, 1977; Vallet et al.,1990 , Spencer et al., 1995). The negative influence of progesterone on progesterone receptors is believed to be due to an inactivation rate of the receptor (Milgrom et al., 1973; Leavitt et al., 1977).

It has been demonstrated that estrogen and progesterone receptor expression differs within cell types in the endometrium of many different species (Milgrom et al., 1973; Luu et al., 1975; Hild-Petito et al., 1988; Spencer et al., 1995; Meikle et al., 2001). The patterns of steroid gene expression within the ovine endometrium were found to be markedly different from those in the myometrium (Spencer and Bazer,

1995). Spencer and Bazer (1995) concluded that expression of uterine estrogen and progesterone receptors are differentially regulated in both a temporal and a spatial manner during the estrous cycle and early pregnancy of the ewe. Alterations in tissue- and cell-specific expression of uterine estrogen and progesterone receptors during the estrous cycle and early pregnancy cannot be attributed solely to changes in systemic steroid hormone concentrations. Locally produced, paracrine-acting factors are most likely involved in regulating ovine uterine estrogen and progesterone receptor gene expression (Spencer and Bazer, 1995). The use of immortalized cell lines possessing nuclear estrogen and progesterone receptors in a co-culture system is emerging as a technique for use in experiments that examine steroid hormone directed epithelial-stromal-myometrial interactions. These immortalized cells maintain functional properties and characteristics of their *in vivo* counterparts (Johnson et al., 1999; Arnold et al., 2001).

Meikle et al. (2001) evaluated the cervix, uterus, and oviduct of prepubertal lambs for the presence of estrogen and progesterone receptors. At 2 months of age, following limited exposure (3 days) to estrogen (1 μg/kg/day) and progesterone (0.3 mg/kg/day), the lambs were shown to have high levels of both estrogen and progesterone

receptors within the cervix and oviduct. There was no significant effect of additional estrogen or progesterone treatment on cervix or oviductal levels of steroid receptors, but treatment did increase mRNA of both estrogen receptor-α and progesterone receptor within the cervix and uterus (Meikle et al., 2001). This is the first report of sex steroid receptors in the oviduct of the immature ewe. Because treatment caused changes in mRNA expression, the receptor proteins are considered functional (Meikle et al., 2001). It appears that gonadal steroids regulate estrogen and progesterone receptor expression differently along the reproductive tract in female lambs, suggesting that peripheral steroid target tissues can modulate responses to the same systemic levels of steroid hormones.

The Ovary

Research conducted by Hild-Petito et al. (1988) evaluated the distribution of estrogen and progesterone receptors within the non-human primate ovary throughout the menstrual cycle. The germinal epithelium of ovaries at all stages of development was found to possess

estrogen and progesterone receptors. Interestingly, estrogen receptors were not detected in any other ovarian structure while progesterone receptors were detected in the stromal and interstitial tissue of the monkey ovary. Progesterone receptors were also detected in theca interna and externa of healthy and atretic follicles within the monkey ovary.

The functional primate corpus luteum possesses nuclear progesterone receptors, which disappear upon luteal regression, thus suggesting that the luteal phase influences the percentage of receptor positive nuclei in the corpus luteum (Hild-Petito et al., 1988). To quantitatively evaluate the progesterone receptors present in the primate corpus luteum, Slayden et al. (1994) utilized radioligand-binding. Their data provided the first evidence of high affinity binding of progesterone to receptors within primate luteal tissue. In contrast to the data collected by Bramley and Menzies (1988a) which suggested a high capacity, low affinity site within the human luteal tissue, the data generated by Slayden et al. (1994) demonstrated that binding by progesterone receptors within the non-human primate luteal tissue is low capacity and high affinity. Ultimately, these data suggest a receptor-mediated autocrine or

paracrine role for progestins, but not estrogens in the primate ovary (Hild-Petito et al., 1988; Slayden et al., 1994).

The Anterior Pituitary

Estrogen increases steady-state levels of progesterone receptor mRNA and progesterone receptor protein in cultured rat gonadotropes (Turgeon et al., 1999). Exposure to increasing progesterone levels results in a rapid, but transient, down-regulation of progesterone receptor mRNA and a change in progesterone receptor protein distribution in these cells (Turgeon et al., 1999).

Maternal Recognition and Maintenance of Pregnancy

Pregnancy recognition in ruminants requires a critical interaction between the conceptus and the endometrium to ensure the continued maintenance of the corpus luteum and the production of progesterone. Inhibition of oxytocin receptors by progesterone is required in order for

pregnancy to become established and sustained (Lamming et al., 1995, Spencer and Bazer, 1995).

The presence of a conceptus has been shown to exert a locally suppressive effect on endometrial oxytocin receptors (Lamming et al., 1995; Spencer et al., 1995; Spencer and Bazer, 1995). Ruminant blastocysts secrete the cytokine, interferon-τ, which acts directly on endometrial epithelial cells, preventing the up-regulation of oxytocin receptors in late luteal phase (Horn et al., 1998; Ivell et al., 2000). During early pregnancy, the level of oxytocin receptors in endometrium remain very low (Ivell et al., 2000). Secretion of interferon-τ acts to enhance the effect of progesterone in suppressing the effect of estrogens upon the oxytocin receptor (Lamming et al., 1995; Spencer et al., 1995).

In the pregnant ewe, on day 16 after fertilization, the oxytocin receptor levels were 45 ± 11 fmol/mg protein in the pregnant horn and 585 ± 131 fmol/mg protein in the non-pregnant horn (Lamming et al., 1995). As expected the pregnant horn had high antiviral activity (interferon-τ) and low oxytocin receptor concentrations whereas the nonpregnant horn had the opposite (Lamming et al., 1995). These data support the possibility that reduced estrogen receptor function mediates

the inhibitory effect of interferon-τ on oxytocin receptor expression (Lamming et al., 1995). Presence of a conceptus in the pregnant horn of a ewe locally suppressed the development of estradiol and oxytocin receptors, but had no apparent effect on the progesterone receptor concentrations (Lamming et al., 1995; Spencer et al., 1995; Wathes et al., 1996). In the rat, progesterone receptors are present only in the myometrium of the pregnant horn, not in the epithelium (Lamming et al., 1995).

Progesterone receptor mRNA is localized to ovine caruncular tissues on day 30 and is undetectable by day 34, while estrogen mRNA is localized to caruncular tissue from days 13 to 30 and in the maternal villi and placentome capsule between days 45 and 70 (Leung et al., 1998). Estrogen receptor protein is barely detectable in either the maternal villi or placentome capsule on days 35 to 40, it is at its highest on day 45, and then returns to basal levels for days 132 to 137 (Leung et al., 1998). The placentome capsule is a layer of maternal connective tissues rich in collagen and fibroblast cells with detectable oxytocin receptors at around day 35 of pregnancy in the ewe (Leung et al., 1998). Progesterone acting via progesterone receptors may suppress oxytocin receptor expression in the placentome during early pregnancy. It is also

possible that oxytocin receptor up-regulation in the placentome capsule may not involve the estrogen receptor.

While the mechanistic pathway by which interferon-τ affects estradiol receptor expression and subsequent oxytocin receptor activity is yet undefined, the necessity of conceptus recognition proteins is clear. Ultimately, the products of the conceptus appear to regulate, either directly or indirectly, steroid receptor expression in the endometrium, which in turn regulates oxytocin receptors.

CHARACTERISTICS OF THE PLASMA MEMBRANE

The plasma membrane is comprised of lipid bilayers that are composed of phospholipids, proteins, cholesterol, glycolipids, and glycoproteins (Singer and Nicolson, 1972; Alberts et al., 1994). The phospholipids are responsible for the majority of the total weight of the plasma membrane with the individual components being: phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, and phosphatidylinositol (Alberts et al., 1994). Phospholipids are amphipathic, having a hydrophilic polar end and a hydrophobic nonpolar

end with a glycerol backbone. They tend to aggregate with the hydrophobic tails inward and hydrophilic head groups outward, thus forming either micelles or sheets (Alberts et al., 1994).

Cholesterol is responsible for approximately 17% of the total weight of the plasma membrane, realizing there do exist between tissue variations. There is a greater ratio of cholesterol to phospholipids in the plasma membrane versus the inner membranes of the endoplasmic reticulum and various other organelles. Membrane fluidity and the mechanical stability of the membrane bilayer are determined, in part, by the amount of cholesterol in the plasma membrane. Cholesterol functions to prevent phospholipids from packing too closely together and crystallizing (Alberts et al., 1994). Membrane fluidity is essential to facilitate the movement of receptors and other molecules within the membrane. A putative cholesterol-docking site has been located on the extracellular surface of the oxytocin receptor found in human embryonic kidney (HEK) cells transfected with the human oxytocin receptor (Gimpl et al., 2000). The docking site is localized between transmembrane helices 5 and 6. This may be a cholesterol specific site because the inactive cholesterol analogue, 4-cholestene-3-one did not interact with this site in the same manner as cholesterol (Gimpl et al., 2000). Ligand

binding of oxytocin receptors was weakly correlated to membrane fluidity (Gimpl et al., 2000). Membrane temperature also contributes to the physical state of the membrane; i.e., fluid or crystalline state. Cholesterol decreases phase transition temperature due to less phospholipid interaction. Cholesterol has also been shown to stabilize the oxytocin receptor in its high-affinity state; however, the loss of receptor stability was not accompanied by changes in membrane fluidity (Gimpl et al., 2000).

BIOCHEMICAL CHARACTERISTICS OF OXYTOCIN

The neurohypophysial hormone oxytocin is a nonapeptide hormone that is essential for reproductive function (Gimpl and Fahrenholz, 2001). This nonapeptide is constructed of the following linked amino acid residues: Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly(NH_2), possessing a disulfide bridge between Cys residues 1 and 6, resulting in a 6 residue cyclic section and a COOH-terminal α amidated three-residue tail (Gimpl and Fahrenholz, 2001). This peptide is primarily released from the posterior pituitary, but is also synthesized and

secreted within peripheral tissues such as the uterus (sow, mare), placenta, and corpus luteum of the ruminant, sow, and primate (Flint and Sheldrick, 1982; Gimpl and Fahrenholz, 2001).

In order for oxytocin to exert an effect, it must bind to its receptor within the target tissue. The oxytocin receptor is a typical member of the rhodopsin type (class I) G-protein-coupled-receptor family (Gimpl and Fahrenholz, 2001). Recent reviews investigating the structure of the oxytocin receptor illustrated that the serpentine receptor creates a cavity between its amino terminus and extracellular loops (Ivell et al., 2001; Gimpl and Fahrenholz, 2001). The oxytocin peptide exhibits the closest contact to the receptor within the space between the N terminus and the first extracellular loop of the receptor (Ivell et al., 2001; Gimpl and Fahrenholz, 2001).

CLASSICAL STEROID HORMONE ACTION

Upon interaction with the target cell, steroids diffuse through the plasma membrane (Baulieu et al., 1971). The steroid then moves across the cytoplasm to the nuclear membrane. While the mechanism of

movement is not known, it is likely chaperoned by another protein (Falkenstein et al., 2000). Once it enters the nucleus of the cell, the steroid binds to a specific receptor that induces a conformational change in the hormone-receptor complex (Carson-Jurica et al., 1990). The conformational change allows for the hormone-receptor complex to bind to a steroid response element located in the 5' promoter region of a particular gene. These response elements will enhance or inhibit gene transcription depending on the cell type (Carson-Jurica et al., 1990). If the response element triggers transcription, it will result in the formation of pre-mRNA; and subsequently introns are removed and mRNA is transported to the rough endoplasmic reticulum for translation to a protein (Falkenstein et al., 2000).

Following binding of the hormone, the responses of the target cells can be quite dramatic. There is variation amongst target cells, but responses can be classified as either short term (1-6 hours) or long term (6-48 hours) (Falkenstein et al., 2000). Examples of short-term responses to estrogen would be increases in hyperemia, imbibition of water, glucose oxidation, lipid synthesis, RNA polymerase activity, and incorporation of precursors into RNA and proteins (Mueller et al., 1958). Long-term responses would include cell hypertrophy and hyperplasia, as

well as increased DNA synthesis, protein synthesis, and stimulation of RNA polymerase activity.

GENOMIC ACTIONS OF STEROIDS

The promoter region of the oxytocin gene possesses a composite hormone response element at ~-160bp (Gimpl and Fahrenholz, 2001). This region is fairly well conserved over five species including the bovine, ovine, mouse, rat and human (Gimpl and Fahrenholz, 2001). The oxytocin gene promoter region of the rat and human does contain a nucleotide sequence nearly identical to the classic estrogen response element (Gimpl and Fahrenholz, 2001). However, the bovine and ovine oxytocin gene promoters are endowed with a response element for steroidogenic factor-1(SF-1) but lack the complete hexanucleotide (Gimpl and Fahrenholz, 2001). The oxytocin receptor gene of the rat has been shown to possess a partial estrogen response element in its upstream promoter, however, it is not functional (Bale and Dorsa, 1997). The bovine oxytocin receptor gene is also believed to possess an

estrogen response element, however, it too appears nonfunctional (Ivell et al., 1998).

The oxytocin gene has a response element in the promoter region, which has been shown to bind and respond to hormone receptors, such as thyroid and retinoic acid receptors, as well as orphan receptors such as SF-1 (Ivell and Walther, 1999). The progestin antagonist, mifepristone (RU 486) has been shown to completely inhibit oxytocin gene transcription within cultured bovine granulosa cells, thus reinforcing the relationship between progesterone receptors and oxytocin at the level of the genome (Lioutas et al., 1997).

CONCEPT OF NONGENOMIC ACTIONS OF STEROIDS

Nongenomic actions are rapid effects of steroid hormones that have been demonstrated on cell membranes or cytoplasmic signal transduction pathways and occur without changes in gene transcription or protein synthesis (Revelli et al., 1998). The ability of steroid hormones to directly interact with receptors of other classes of biochemicals presents an additional example of non-traditional steroid activity. The

range of interactions is varied, as are the effects upon the target tissues, and the ultimate response of the system. Steroid/peptide receptor cross-talk presents a novel area of investigation into the mechanism of hormone action.

Multiple reviews regarding nongenomic interaction between steroid and peptide hormones have concluded that there is evidence that steroid hormones use multiple cell-signaling pathways to exert an effect on a target cell response (Revelli et al., 1998; Moore and Evans, 1999; Falkenstein et al., 2000). Some of the first data demonstrating an interaction between a steroid and a G-protein-coupled receptor were presented by Zingg et al. (1998). Utilizing progesterone and oxytocin, they demonstrated that progesterone represents an endogenous high-affinity nonpeptide antagonist of the rat oxytocin receptor (Zingg et al., 1998). These data are in agreement with those of Bogacki et al. (2002), who demonstrated that within bovine endometrial plasma membranes, progesterone inhibits the binding of oxytocin to its receptor. While the precise mechanism of action for progesterone-induced inhibition of oxytocin binding to its receptor is still unknown, a direct interaction at the membrane level has been hypothesized (Zingg et al., 1998; Grazzini et al., 1998; Bogacki et al., 2002). Grazzini et al. (1998) demonstrated that

progesterone inhibits oxytocin binding to the oxytocin receptor *in vitro* and binds with high affinity to CHO cells expressing the rat oxytocin receptor. Other specific effects of progesterone on the oxytocin receptor include suppression of inositol phosphate production and calcium mobilization (Grazzini et al., 1998, Zingg et al., 1998). In experiments conducted by Burger et al. (1999) high concentrations of progesterone were able to attenuate signal transduction of many G-protein-coupled receptors, but this effect was more cell-specific than receptor specific.

In addition to understanding the processes by which differing classes of hormones interact, the discovery of actual membrane steroid receptors in a variety of species and tissue types has also received a great deal of attention. A membrane receptor for glucocorticoids has been detected in the amphibian brain (Evans et al., 1998; 2000). This receptor has been shown to possess a high affinity binding with a great degree of stablity (Evans et al., 1998). Increasing evidence for the existence of multiple estrogen and progestin membrane receptors will be discussed in future sections.

Membrane Estrogen Receptor

A critical function of the traditional estrogen receptor may be to link the estrogen receptor to phosphatidylinositol-3-OH kinase, because increased levels of membrane and cytoplasmic estrogen receptors have been demonstrated after estrogen stimulation in human vascular endothelial cells (Simoncini et al., 2000). It is likely that phosphatidylinositol-3-OH kinase is being reserved and activated by a small subset of ligand-bound, membrane associated estrogen receptors (Simoncini et al., 2000).

The signaling pathway mediating the rapid action of estrogen involves the interaction of Shc adaptor protein with estrogen receptor-α, Shc protein activation and the phosphorylation of mitogen activated protein (MAP) kinase. The association between estrogen receptor-α and Shc is direct. Data suggest that estrogen receptor-α can mediate the rapid effects of estrogen on Shc, MAP kinase, Elk-1, and morphological changes in breast cancer cells (Song et al., 2002).

It is also possible that there is a receptor for estradiol-17β present on the surface of neuroblastoma cells that can transduce signals intracellularly via activation of MAP kinase signaling pathway. The

hormone estradiol-17β mediates its effect on the MAP kinase signaling pathway via action at the neuronal membrane, which is a novel mechanism of estrogen action (Watters et al., 1997).

Transfection of estrogen receptor-α into CHO cells resulted in a single transcript expressing estrogen receptor in nuclear and membrane fractions (Razandi et al., 1999). Transfection of estrogen receptor-β also yielded nuclear and membrane fractions. While the membrane concentrations of both α and β receptor forms were significantly lower than their nuclear concentrations, they both exhibited similar binding affinities and possessed the ability to activate G proteins, extracellularly regulate kinases, and promote cell proliferation (Razandi et al., 1999).

Knowing the structural relationship between the plasma membrane and nuclear populations of estrogen receptor-α is important to understanding the effectiveness of the receptors (Razandi et al., 1999; Norfleet et al., 2000). Using a subclone (F10) of the rat pituitary tumor cell line (GH_3/B6), known to express high levels of membrane estrogen receptor-α, Norfleet et al. (2000) have confirmed the structural relationship between the membrane and nuclear receptor populations. It has also been demonstrated by Norfleet et al. (2000) that antibodies raised against estrogen receptor-α recognize both the membrane and

nuclear form of the receptor. The ability of the antibodies to modulate a biological response through their binding to estrogen receptor α reveals a functional role for those receptors and suggests that alteration of receptor conformation or aggregation is involved in the rapid membrane estrogenic response (Norfleet et al., 2000). Both CHO cells transfected with estrogen receptor-α cDNA, and GH_3/B6/F10 cells express estrogen receptor-α on the plasma membrane, as well as in the nucleus, and exhibit a rapid response to estrogen (Razandi et al., 1999; Norfleet et al., 2000).

Membrane Progesterone Receptor

There may exist a family of membrane proteins in gonadal tissues that bind progesterone and function as progesterone receptors. Godeau et al. (1978) demonstrated a nongenomic effect of progesterone, on *Xenopus* oocyte maturation, by comparing a polymer-bound steroid applied to an egg externally with an internally microinjected polymer-linked steroid. The steroid, applied externally, activated the maturation process while the solution injected into the oocyte did not. Progesterone

induced *Xenopus* oocyte maturation was not achieved via binding of the steroid to a classical cytosolic steroid receptor (Godeau et al., 1978; Bayaa et al., 2000; Lutz et al., 2000; Morrison et al., 2000). Lutz et al. (2000) suggests two different sets of progesterone binding sites on oocyte membranes, low affinity and high affinity. These are thought to correlate to nonspecific and specific binding sites. Progesterone has also been shown to stimulate tyrosine kinase activity associated with the plasma membrane of *Xenopus* oocytes. The tyrosine kinase is responsible for stimulation of phospholipase C activity within *Xenopus* oocytes (Morrison et al., 2000). While there is substantial evidence supporting the role of progesterone in *Xenopus* oocyte maturation, this is the first evidence of progesterone stimulating tyrosine kinase (Godeau et al., 1978; Morrison et al., 2000).

Progesterone can cause rapid, GTP-dependent inhibition of adenylyl cyclase in washed oocyte membranes (Lutz et al., 2000). This lends support to the idea that the progesterone receptor may be localized to the membrane and suggests that the plasma membrane progesterone receptor is a seven-pass G-protein-coupled-receptor like the oxytocin receptor (Sadler and Maller, 1981;1985).

The research of Bagowski et al. (2001) provides evidence for the involvement of the classical progesterone receptor in meiotic maturation as evidenced by its interaction with p42 MAP kinase and phosphatidylinositol-3-OH-kinase. Classical steroid receptors have dual functions as transcription activators in the nucleus and as signal transducers outside of the nucleus (Bayaa et al., 2000; Bagowski et al., 2001; Boonyaratanakornkit et al., 2001). The cloned *Xenopus* membrane progesterone receptor provides an example of just such a dual-purpose receptor because it is also the oocyte receptor responsible for progesterone-induced oocyte maturation (Bayaa et al., 2000). Another dual-purpose receptor is proposed by Boonyaratanakornkit et al. (2001) who demonstrated that both isoforms of human progesterone receptors interact directly and efficiently with a subset of Src homology 3 (SH3) domains in proteins. These data suggest that the progesterone receptor-SH3 domain interaction is largely hormone-dependent *in vitro* and *in vivo* and that direct binding to SH3 domains is a unique property of progesterone receptors (Boonyaratanakornkit et al., 2001). A suggested mechanism of action is that ligand interaction at the carboxy terminus induces a conformational change at the N terminus that somehow exposes the proline rich SH3 interaction motif on the receptor.

The progesterone receptor only interacts with the left-handed polyproline helix. The interactions of estrogen and progesterone receptors with Src kinase *in vivo* were analyzed and demonstrated that estrogen and progesterone receptors can interact with Src kinase simultaneously through independent binding to SH2 and SH3 domains. However, *in vitro*, the progesterone receptor is apparently capable of binding to and activating Src kinases independently of the estrogen receptor (Boonyaratanakornkit et al., 2001). The exact manner in which estrogen and progesterone receptors cooperate to modulate Src kinase activity is unknown; the hypothesis of Boonyaratanakorkit et al. (2001) states that the progesterone receptor functions as a dual-protein capable of activating signaling pathways in the cytoplasm through hormone-dependant interaction with the regulatory SH3 domain of Src kinases directly while maintaining its traditional role in the nucleus acting as a hormone-dependent transcription factor. Basically, the progesterone receptor, existing in the cytoplasm, acts on the SH3 domain that is coupled to SH2 and the Src kinases, and by doing so activates the Ras/Raf-1 MAP kinase pathway to activate a nuclear transcription factor. The same cytoplasmic progesterone receptor also acts as a

traditional receptor, by forming a hormone-receptor complex acting at the level of the nucleus.

Experimental evidence from studies conducted by Peluso et al. (2001) demonstrates the existence of a 60 kDa progesterone binding protein that functions as a low-affinity, high–capacity membrane receptor for progesterone within rat granulosa cells. Additional evidence for a membrane progesterone receptor is provided from the research of Krebs et al. (2000) who have discovered the membrane associated 25-Dx progesterone binding protein, the rat homolog of the human membrane associated progesterone binding protein Hpr6.6. The progesterone binding protein 25-Dx is repressed by the activation of the nuclear progesterone receptor within the hypothalamus and has been shown to bind to other steroids including corticosterone, testosterone, cortisol, and promegestone with varying affinities (Krebs et al., 2000).

Human spermatozoa are yet another cell type that is known to possess a plasma membrane progesterone binding site (Blackmore and Lattanzio, 1991; Meizel 1991; Ambhaikar and Puri, 1998; Luconi et al., 1998). This progesterone receptor is localized on the head of the human spermatazoa; it is believed that when bound, it acts to elicit an influx of calcium ions that are involved in the induction of the acrosomal

reaction required for capacitation (Blackmore and Lattanzio, 1991; Meizel 1991; Blackmore et al., 1996; Luconi et al., 1998). The membrane progesterone binding site is very steroid specific, and unlike sites in female tissues, the antiprogestin mifepristone is an ineffective inhibitior of progesterone binding (Blackmore and Lattanzio, 1991).

Membrane bound progesterone binding sites have also been identified within the corpora lutea of many species, including the bovine (Rae et al., 1998; Menzies et al., 1999), ovine (Bramley and Menzies, 1994), human (Bramley and Menzies, 1988a), equine (Bramley et al., 1995) and porcine (Bramley and Menzies, 1988b). These sites were all shown to demonstrate high affinity binding of progesterone, suggesting that there are steroid receptors located at the level of the plasma membrane.

G-PROTEIN-COUPLED RECEPTORS

In recent years there has been extensive research conducted to evaluate G-protein-coupled receptors and their role within the biological system. There is considerable evidence for interactions both among and

within members of the G-protein-coupled receptor family. Those interactions have been shown to exist as homodimerization, heterodimerization, oligomerization, and heterooligomerization.

The single largest family of transmembrane receptors involved in cell signaling is the G-protein-coupled receptor superfamily (Salahpour, 2000). One of the three primary classes of membrane receptors, they are responsible for associating with the G-protein complex and affecting a multitude of biological actions within the cell.

It was originally believed that G-protein-coupled receptors functioned as monomers, and that the stoichiometry of the receptor, G-protein, and effector was 1:1:1 (Bourne et al., 1990). However, there has also been considerable evidence collected to suggest that they could function as dimers (Conn et al., 1982; Hazum et al., 1985; Hebert et al., 1996; Cvejic and Devi, 1997). Research conducted by Maggio et al. (1993) provided an early example of intermolecular interactions between two receptors. Functional receptors were formed from coexpressing chimeric α_2 adrenergic and M3 muscarinic receptors and it was observed that no activity occurred when the receptors were expressed alone.

Within rat gonadotropes and in a lactotrope-derived cell line expressing the GnRH receptor (GGH_3) it has been demonstrated that

GnRH receptors couple to multiple G proteins, specifically $G_i\alpha$, $G_s\alpha$, and, $G_{q/11}\alpha$ (Stanislaus et al., 1998). The abiltiy of the GnRH receptor to couple to multiple G proteins allows the GnRH receptor to activate multiple signalling pathways, which may allow for the stimulation of multiple gonadotrope responses (Stanislaus et al., 1998). The utilization of both the rat gonadotropes and the transfected cell line further demonstrates that the dimerization of the GnRH receptor with multiple G proteins is not gonadotrope specific, but rather receptor specific (Stanislaus et al., 1998).

G-Protein-Coupled-Receptor Homodimerization

The functional and physical interactions of G-protein-coupled receptors as homodimers were evaluated in 1982 by Conn et al., who investigated the GnRH receptor and its microaggregation, while Hebert et al. (1996) more recently used the β_2-adrenergic receptor as a model for homodimerization.

GnRH Receptors

In the case of GnRH it appears that it is possible for the effector (LH release) to be activated by the microaggregationn of the plasma membrane receptors (Conn et al., 1982). Utilizing rat pituitary cells maintained in culture, these investigators demonstrated that a GnRH agonist prepared as a dimer is effective at stimulating LH release from pituitary cultures, and when incubated with an antibody against the agonist, the stimulatory effect is enhanced. The antibody-dimer conjugate was successful in cross-linking the receptors in the lateral plane of the membrane (Conn et al., 1982).

β_2-adrenergic Receptors

The β_2-adrenergic receptor is a member of the rhodopsin family that has been shown to exist as a dimer (Hebert et al., 1996). Activation of the β_2-adrenergic receptor results in the stimulation of an exchange of GDP/GTP within the alpha subunit of the stimulatory G protein to which the β_2-adrenergic receptor is coupled. This results in the activation of the

effector protein, adenylyl cyclase, and ultimately results in the increases of the second messenger, cAMP. The β_2-adrenergic receptor undergoes agonist stimulated clustering and internalization through clathrin-coated pits and vesicles (Hebert et al., 1996; Angers et al., 2000). The COOH-terminal tail plays a role in the receptor down-regulation, as it is involved in mediating desensitization through both second messenger-dependent and independent kinases (Cvejic et al., 1996).

Research conducted by Hebert et al. (1996) and Angers et al., (2000) illustrates that β_2-adrenergic receptors can form homodimers. Evidence has been presented demonstrating that agonist stimulation of the β_2-adrenergic receptor stabilized the dimerized receptor while inverse agonists, such as timolol, favored the monomeric form. This suggests that interconversion between monomer and dimer may be important for biological activity (Hebert et al., 1996). The ability of a peptide derived from TM VI (transmembrane domain VI), to alter the relative amounts of the β_2-adrenergic receptor dimer, illustrates the possibility of a dynamic equilibrium existing between the monomeric and dimeric forms (Hebert et al., 1996). There is also an interference with the β_2-adrenergic receptor-signaling pathway that occurs when the equilibrium shifts away from the dimer (Hebert et al., 1996). Thus, there is a case presented for

receptor-specific dimerization determinants, because the β_2-adrenergic receptor did not form a heterodimer with the M2 muscarinic receptor, which has been known to homodimerize (Hebert et al., 1996).

Sigma-opioid Receptors

Research conducted by Cvejic and Devi (1997) provided the first evidence for homodimerization of sigma-opioid receptors. Using crosslinking and immunoprecipitation methods similar to those employed by Hebert et al. (1996), Cvejic and Devi (1997) demonstrated that sigma-opioid receptors do exist as dimers and that the level of dimerization is agonist dependent and requires an intact C-terminal tail (Cvejic et al., 1996; Cvejic and Devi, 1997).

G-Protein-Coupled-Receptor Heterodimerization

While there is well established evidence suggesting that G-protein coupled-receptors exist as homodimers, their ability to function as

heterodimers has only recently been explored. The possibility that heterodimers of G-protein coupled-receptors exist and exhibit distinct characteristics has potentially huge clinical applications (Evans et al., 2000; Gomes et al., 2000). It is known that the $GABA_{-B}$ receptor functions as a heterodimer and that formation of a functional receptor from two nonfunctional receptors suggests that dimerization is necessary (White et al., 1998). The M3-muscarinic receptor and the δ-opioid receptor are two additional examples of G-protein coupled-receptors that form heterodimers (Zeng and Wess, 1999; Jordan and Devi, 1999). Heterodimerization is possibly another mechanism by which cells modulate specific cellular signalling and function.

Members of the somatostatin receptor family offer the unique property of forming both homo- and heterodimers (Rocheville et al, 2000). The heterodimerization that occurs amongst the five subtypes of the somatostatin receptor family is considered to be restricted to specific receptor subtype configurations (Rocheville et al, 2000). It has been shown that heterodimerization between somatostatin receptor subtype 1 and somatostatin receptor subtype 5 does alter the functional properties of the resulting receptor, specifically affecting ligand binding, affinity and agonist-induced receptor internalization and up-regulation in CHO-K1

cells (Rocheville et al., 2000). The research of Jordan and Devi (1999) utilizing HEK 293 cells, COS-7 cells, and CHO cells also illustrated that heterodimerization of the functional opioid receptors, κ and δ, results in a new receptor that exhibits altered ligand binding, as well as modified functional properties. The latter was the first direct evidence for the heterodimerization of two opioid-receptor types.

Opioid receptors can be classified into three main cell types: δ,μ and κ (Law et al., 1982). The opioid peptide-receptor system is known to regulate a variety of biological functions related to perception of pain, locomotion, motivation, autonomic function, immunomodulation and hormone secretion (George et al., 2000). Differing from the β_2-adrenergic receptor, in terms of G protein activity, activation of the sigma and kappa opioid receptors results in activation of the inhibitory regulatory unit of the alpha subunit of the G protein. Thus, there is a decrease in adenylyl cyclase activity, as well as a decrease in the second messenger cAMP. The opioid receptors activate the MAP kinases; additionally, agonist binding does cause rapid internalization and down-regulation of the receptors (Trapaidze et al., 2000). A key component in agonist-mediated down-regulation is the Thr^{353} located in the COOH-terminal tail of the δ opioid receptor (Cvejic et al., 1996).

The research of George et al. (2000) provided the first evidence for the direct interaction for δ and μ opioid receptors to form oligomers within COS-7 cells and CHO cells. The oligomers formed by the δ and μ opioid receptors expressed novel pharmacological and G protein coupling properties (George et al., 2000).

STATEMENT OF THE PROBLEM

Biological systems require many complex interrelationships for the regulation of their activity. The steroid hormone, progesterone, and the peptide hormone, oxytocin, provide an example of two different classes of hormones operating within the same target tissues to accomplish different tasks. Progesterone and oxytocin play essential roles in reproductive processes, by functioning to regulate the female reproductive cycle, pregnancy, and parturition. The full extent of these interactions are not yet completely understood, nor are the mechanisms by which these hormones act.

The nuclear-mediated actions of steroids have been well defined, however, the occurrence of steroid mediated rapid-responses within target tissues suggests the possibility of nongenomic activity for this class of biochemicals. Steroids may posses the ability to bind directly to the receptor of a peptide hormone, or a closely associated protein. These possible nongenomic actions of steroids provide multiple examples of alternative mechanisms of action or complementary roles of hormones within a system.

An evaluation of the possible nongenomic actions progesterone may have with the oxytocin receptor within the ovine uterine plasma membranes would provide a more detailed picture of the "cross talk" occurring between these hormones. Essentially, any observations collected regarding this complex interrelationship will help to clarify the role of these hormones in the reproductive process and may lead to the development of new contraceptives for humans or to improved methods of estrous cycle control in domestic livestock.

INTRODUCTION

The ruminant (e.g. cattle and sheep) uterus contains oxytocin (OT) receptors, which when bound by endogenous oxytocin provoke the pulsatile release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) that may promote regression of the corpus luteum during the estrous cycle (McCracken et al., 1984; Bazer et al., 1998). Ovarian steroids are responsible for the regulation of the concentration of oxytocin receptors (Sheldrick and Flint, 1985; Zhang et al., 1992). This action is presumably accomplished via genomic actions of these steroids. During diestrus, progesterone (P_4) acts to down-regulate oxytocin and estrogen receptors, and eventually, by an autoregulatory mechanism, down-regulates its own receptor (Zhang et al., 1992). Exposure of the uterus to increasing follicular estrogen during proestrus and estrus up-regulates estrogen receptor and oxytocin receptor gene expression (Bazer et al., 1998, Zingg et al., 1998).

Progesterone treatment has been shown to down-regulate endometrial oxytocin receptors by suppressing the transcription of the oxytocin receptor gene (Stewart et al., 1993; Hazzard et al., 1998). More recently Grazzini et al. (1998) has shown that progesterone may be

acting via a nongenomic mechanism to inhibit the binding of oxytocin to its receptor in the rat uterus, which is consistent with evidence that steroids may act at this level in other systems. The existence of signal-generating steroid receptors in the plasma membrane of several cell types has been shown in recent experiments (Revelli et al., 1998). Progesterone has been found to bind to a membrane receptor in rat granulosa cells (Peluso et al., 2001), oocytes of *Xenopus laevis* (Godeau et al., 1978) and human sperm (Meizel et al., 1991). Estrogen receptors have been detected in plasma membranes of transfected Chinese Hamster Ovarian (CHO) cells (Razandi et al., 1999) and pituitary tumor cells (Norfleet et al., 2000). Thus, steroids may have more than one site of action in target cells. As example, besides regulating oxytocin receptor gene transcription, progesterone may also act at the level of the plasma membrane to ensure uterine quiescence during early pregnancy through inhibition of oxytocin binding to oxytocin receptors.

The objectives of the present research were to examine the *in vivo* and *in vitro* effects of progesterone on the binding of oxytocin to its receptor in the ovine endometrium and to determine whether high affinity binding sites for progesterone are present in the ovine endometrial membranes.

MATERIALS AND METHODS

ANIMALS

Mature Polypay ewes were rendered anestrus by ovariectomy at least 14 days prior to use in the experiments. The animals were anesthetized by use of intravenous injection of sodium pentothal (5%, Abbott Laboratories, North Chicago, IL) followed by maintenance of anesthesia by use of closed circuit inhalation of an oxygen-halothane mixture. The ovaries were removed via a midventral laparotomy. Intramuscular injections of banamine (50 mg/ml, Schering-Plough Animal Health Corp., Union, NJ) and penicillin (300,000 units/ml, Butler Company, Columbus, OH) were administered immediately following surgery. Upon recovery the ewes were assigned to experimental groups. All experimental procedures were performed in accordance with the institutional guidelines for the care and use of animals.

EXPERIMENT 1. OXYTOCIN BINDING TO PLASMA MEMBRANES RECOVERED FROM EWES TREATED *IN VIVO*

To evaluate the *in vivo* effect of P_4 on the binding of OT to its endometrial receptor, 11 ovariectomized (OVX) ewes were assigned to three treatment groups: 1) control (n=4), 2) P_4 (n=4), 3) Mifepristone (RU 486; n=3). To impose an abbreviated estrous cycle, similar to a naturally occurring cycle, all animals received subcutaneous injections of 25 μg of E_2/day for 2 days followed by daily injections of 10 mg of P_4 for 5 consecutive days. The injection schedule (Fig. 1) was one-half as frequent as that used by Vallet et al. (1990). This treatment regimen followed by the injection of E_2 on each of 3 days as described below ensured an enriched population of OTR in the endometrium. Specific daily treatments were imposed as follows during the last 3 days: Grp 1) vehicle (corn oil); 2) 10 mg P_4; 3) 10 mg P_4 + 10 mg RU 486. Endometrium was removed on Day 11 via midventral laparotomy, quick frozen in liquid N_2 and stored at –80°C until OTR assay.

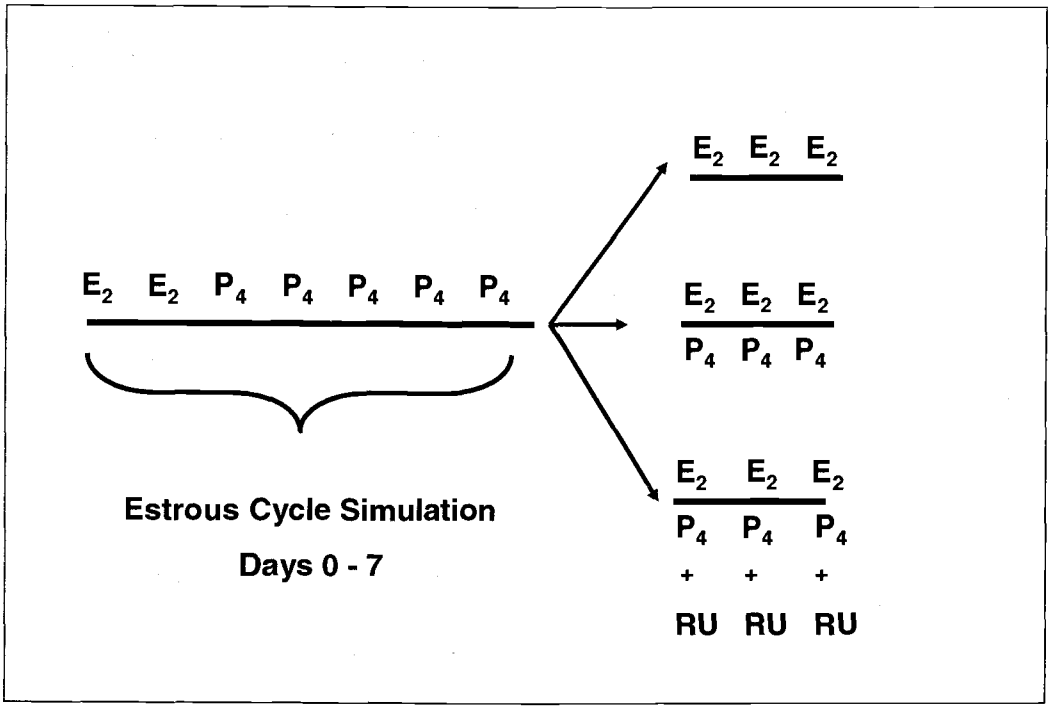

Figure 1. Schedule of sequential daily hormone treatments initiated at least 14 days after ovariectomy. Ewes were assigned randomly to one of three treatment groups. All hormone treatments were given via s.c. injections at 24 h intervals. Each treatment group received 7 days of injections to mimic an abbreviated estrous cycle followed by 3 days of specific hormone treatment (E_2 plus either vehicle, P_4 or P_4 + RU 486) given at 12 h intervals on each day. Tissue was removed via midventral laparotomy 12 h following final injection.

EXPERIMENT 2. *IN VITRO* OXYTOCIN BINDING

To evaluate the effects of P_4 on the binding of OT to its endometrial receptor *in vitro*, endometrium was removed via a mid-ventral laparotomy from a total of nine OVX ewes receiving the control group treatment as described above for the *in vivo* experiment. Upon removal, fresh endometrial tissue was placed in ice-cold saline and transferred to the laboratory where the membrane fraction was isolated by homogenization in buffer (25 mM Tris-HCl, 0.25 M sucrose), subjected to differential centrifugation to remove nuclei (2,000 x g) and mitochondria (20,000 x g), and finally centrifuged at 100,000 x g to recover plasma membranes. Following isolation the membranes (1 mg protein/ml) were distributed to each of three flasks containing 1.5 ml of incubating buffer (25 mM Tris-HCl, 0.01% NaN_3, 15 mM EDTA) with the following additions: Flask 1) vehicle (30 µl absolute ethanol); Flask 2) 5 ng/ml P_4 + 15 µl absolute ethanol; Flask 3) 5 ng/ml P_4 + 10 ng/ml RU 486. Progesterone and RU 486 were each dissolved in 15 µl of absolute ethanol. All samples were incubated for 60 min at room temperature (25°C) as described by Grazzini et al. (1998). The samples were centrifuged (100,000 x g) and the resulting pellet was resuspended in

membrane diluting buffer (25 mM Tris-HCl, 0.01% NaN_3). The suspension was centrifuged (100,000 x g) and the resulting pellet was resuspended in membrane diluting buffer to a concentration of 1.5 mg/ml. Duplicate aliquots of samples from four of the ewes were stored at –80°C until assayed for OT receptors. Duplicate aliquots of membrane collected from the five remaining ewes were immediately subjected to an OT receptor assay. The difference in tissue handling was imposed to evaluate the possibility that receptors may be lost due to freezing and thawing.

EXPERIMENT 3. SPECIFICITY OF PROMEGESTONE BINDING

Endometrial tissue was evaluated for progestin binding by use of a modified radioreceptor binding assay as described below. The ability of other compounds to compete with [^{3}H] promegestone (R5020) for binding to the plasma membrane was also evaluated. Duplicate aliquots of plasma membranes isolated from control animals (n=2) as in Exp. 1 were exposed to a saturating concentration of [^{3}H] R5020 (8nM) and a 200-fold excess of unlabeled competitor (P_4, R5020, E_2, cortisol, testosterone, RU 486, or OT). Data on binding of R5020 in the

presence of competitor are expressed as percentage of [^{3}H] R5020 bound in the absence of competitor.

EXPERIMENT 4. EFFECT OF PROGESTERONE ON PROGESTIN BINDING SITES

To aid in understanding the suppressive effect of P_4 on the OTR, plasma membranes were examined for changes in the concentration of high affinity binding sites for P_4 after exposure to the progestin. An aliquot of plasma membranes isolated from the control animals (n=5) was distributed to each of three flasks, and these were incubated for 1 h at 25°C. Each flask contained 1.5 ml of incubating buffer (25 mM Tris-HCl, 0.01% NaN_3, 15 mM EDTA) with vehicle, P_4 or P_4 + RU 486 as described above for Exp. 2. After incubation plasma membranes were processed as for Exp. 2 and the resulting pellet was resuspended in membrane diluting buffer (25 mM Tris-HCl, 0.01% NaN_3) to a concentration of 1.5 mg protein/ml. Membranes were subjected to a radioreceptor exchange assay by incubation for 18 h at 4° C with 8 nM of [^{3}H]-R5020 or a 200-fold excess of unlabeled R5020 as previously validated in our laboratory (Hazzard and Stormshak, 1997).

Concentration of specifically bound progestin was estimated by subtracting the nonspecifically bound progestin from the total quantity that was bound.

OXYTOCIN RECEPTOR ASSAY

Endometrial tissue was evaluated for OTR binding by use of a modified procedure of Hazzard and Stormshak (1997) adopted from original methods of Mirando et al. (1993). Specific binding of OT to its receptor was determined using [^{3}H]-OT (44.5 Ci/mmol, New England Nuclear, Boston, MA) (.625 to 10nM) and 200-fold excess of unlabeled OT to correct for nonspecific binding. Data from the saturation curve revealed that the population of receptors was saturated at an OT concentration of 5nM (Fig. 2). Thus, this saturating concentration was chosen for routine use in subsequent assays conducted to quantify tissue concentrations of OT receptors. Realizing that this point was slightly greater than that determined by Hazzard and Stormshak (1997) it still was within the range described by Vallet et al. (1990) and thereby acceptable for use. Scatchard analysis (Fig. 3) of the saturation data

depicted in Fig. 2 revealed a K_d value of 1.01 x 10^{-10}M suggesting measurement of a single binding site with high affinity for OT.

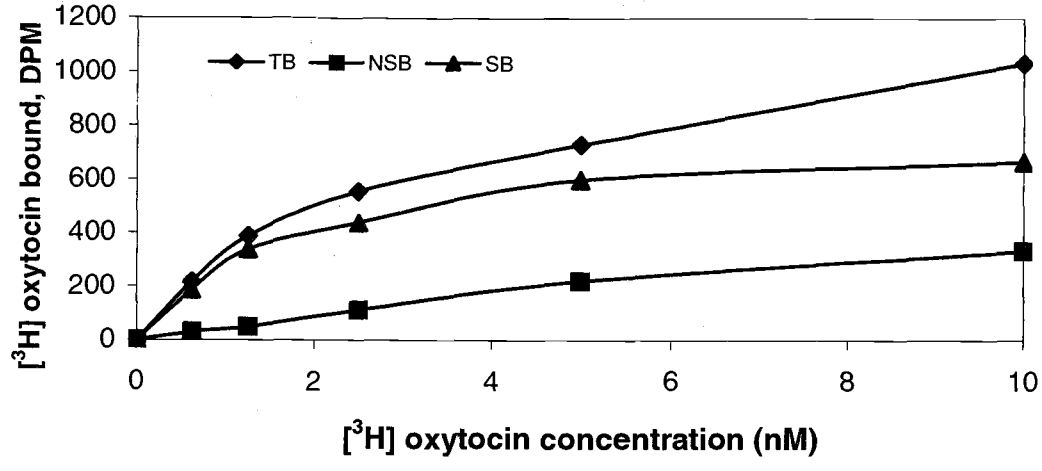

Figure 2. Saturation curve for OT binding to OTR. Duplicate aliquots of plasma membranes (1 mg protein/ml) collected from control ewes (n=5) were subjected to [^{3}H] OT alone or in the presence of a 200-fold excess of unlabeled ligand. Nonspecific binding (NSB) values were subtracted from total bound (TB) to determine specific binding (SB).

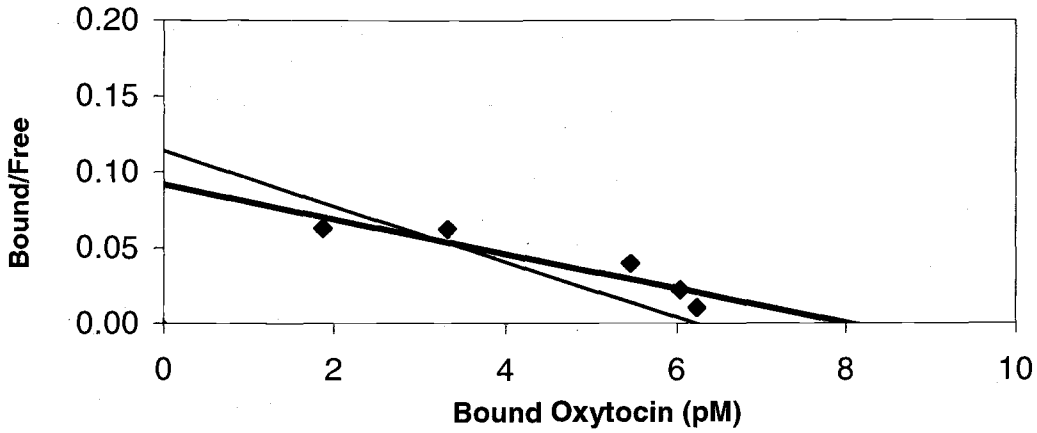

Figure 3. Specifically bound OT values generated from the saturation assay were plotted against the values of Bound/Free for Scatchard analysis. A K_d value of 1.01 x 10^{-10}M was generated.

PROGESTIN BINDING ASSAY

Endometrial tissue collected from control ewes was homogenized in homogenization buffer (25 mM Tris-HCl, 0.25 M sucrose) and subjected to differential centrifugation as described above to recover plasma membranes. Following isolation, the membranes (1 mg protein/ml) were utilized in a P_4 radioreceptor exchange assay. Specific binding of P_4 and R5020 to the plasma membrane fraction was determined using [^{3}H] P_4 (114.4 Ci/mmol, New England Nuclear, Boston,

MA) and [^{3}H] R5020 (84 Ci/mmol, New England Nuclear, Boston, MA) (0.5 to 16 nM) and a 200-fold excess to correct for nonspecific binding. Data from the saturation curve revealed that the population of receptors was saturated at a concentration of 8nM P_4 (Fig. 4) and 8 nM R5020 (Fig. 5). Scatchard analysis revealed high affinity P_4 and R5020 binding sites (K_d 1.2 x 10^{-9} and 1.74 x 10^{-10}M, respectively). The similarities in saturation profiles and Scatchard analysis demonstrated between P_4 and R5020 served as a basis for our use of R5020 in subsequent assays.

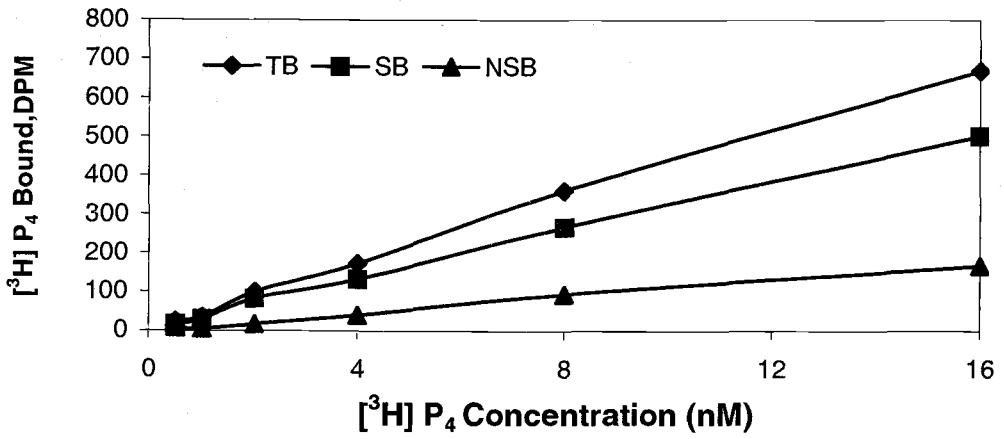

Figure 4. Saturation curve for P_4 binding to plasma membrane. Duplicate aliquots of plasma membranes (1 mg protein/ml) collected from control treated ewes (n=5) were subjected to [^{3}H] P_4 alone or in the presence of a 200-fold excess of unlabeled ligand. Nonspecific binding (NSB) values were subtracted from total bound (TB) to determine specific binding (SB).

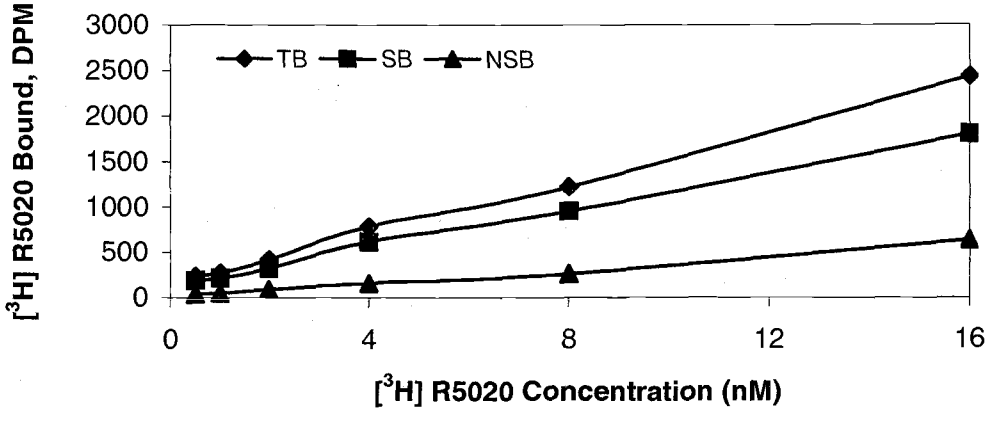

Figure 5. Saturation curve for R5020 binding to plasma membrane. Duplicate aliquots of plasma membranes (1 mg protein/ml) collected from control treated ewes (n=5) were subjected to [^{3}H] R5020 alone or in the presence of a 200-fold excess of unlabeled ligand. Nonspecific binding (NSB) values were subtracted from total bound (TB) to determine specific binding (SB).

STATISTICAL ANALYSIS

All data were subjected to analysis of variance. Differences among means were tested for significance by Student's t-test.

RESULTS

EXPERIMENT 1. OXYTOCIN BINDING TO PLASMA MEMBRANES RECOVERED FROM EWES TREATED *IN VIVO*

In vivo treatment with P_4 significantly suppressed the binding of OT to endometrial OTR (control, 121 ± 18 vs P_4, 40 ± 21 fmol/mg protein; $p<0.01$) while treatment with the antagonist, RU 486, blocked the suppressive effect of P_4 (RU 486, 140 ± 24 fmol/mg protein; $p>0.05$, Fig. 6).

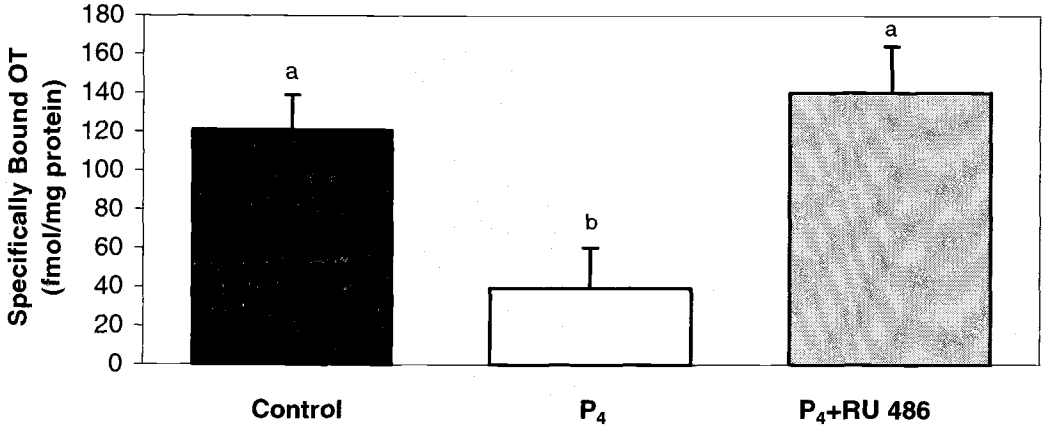

Figure 6. Specifically bound OT in plasma membranes of control and treated ewes. Data represent duplicate aliquots of endometrial plasma membrane samples collected from three experimental groups (n = 3 or 4/group). [a, b] Means (±SE) with different superscript letters differ ($p<0.01$).

EXPERIMENT 2. *IN VITRO* OXYTOCIN BINDING

The *in vitro* effects of P_4 on the binding of OT to its endometrial receptor were similar to those observed when P_4 was administered to the ewes. Samples that were frozen prior to OTR analysis yielded the following data representing OT specifically bound to its receptor (control, 60 ± 5; P_4, 38 ± 5; P_4 + RU 486, 62 ± 9 fmol/mg protein; $p<0.05$, Fig. 7)

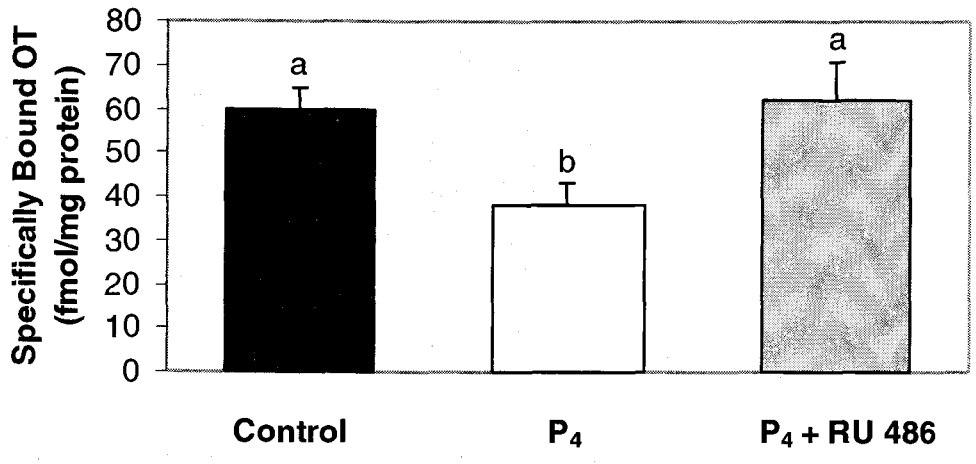

Figure 7. Effect of i*n vitro* exposure to P_4 on the binding of OT to its endometrial receptor. Duplicate aliquots of membrane preparations were incubated with P_4 and P_4 + RU 486. [a, b] Means (±SE) with different superscript letters differ ($p<0.05$).

The five replicate samples that were assayed immediately after membrane incubation also produced a similar binding pattern of OT binding (control, 273 ± 42; P_4, 128 ± 33; P_4 + RU 486, 245 ± 36 fmol/mg protein; $p<0.05$, Fig. 8); however, the concentrations of bound OT were fivefold greater than the samples that were subjected to freezing and thawing prior to OT receptor analysis.

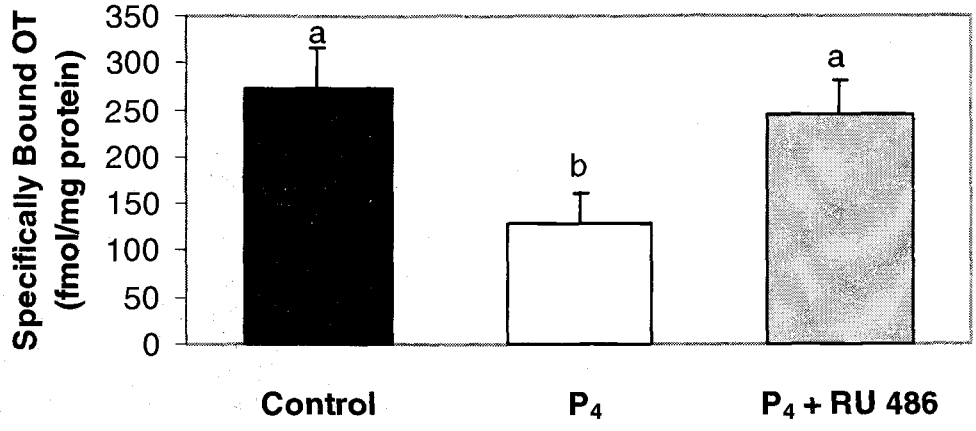

Figure 8. Specifically bound OT in plasma membranes exposed to P_4 alone and P_4 + RU 486. Duplicate aliquots of endometrial plasma membrane samples collected from control ewes immediately assayed after incubation, without freezing and thawing. [a, b] Means (±SE) with different superscript letters differ ($p<0.05$).

EXPERIMENT 3. SPECIFICITY OF PROMEGESTONE BINDING

The ability of specific compounds to compete with [^{3}H] R5020 for binding to the plasma membrane is depicted in Fig. 9. The binding of [^{3}H] R5020 was inhibited by exposure to unlabeled R5020, P_4, RU 486 and OT but not by E_2, cortisol, or testosterone (R5020, 56; P_4, 51; RU 486, 63; OT, 60; E_2, 85; cortisol, 92; testosterone, 89% of [^{3}H] R5020 total bound; $p<0.05$).

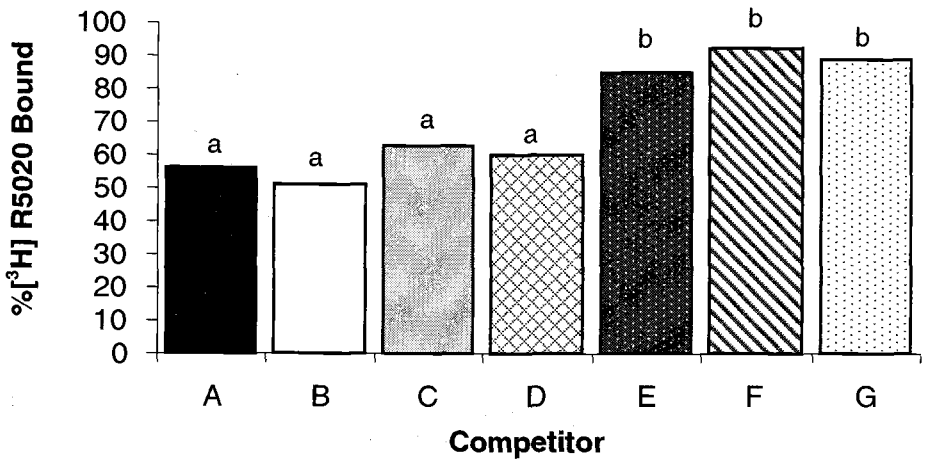

Figure 9. Percentage of maximum [^{3}H] R5020 bound in the presence of selected unlabeled competitors. A) R5020, B) P_4, C) RU 486, D) OT, E) E_2, F) cortisol, G) testosterone. Means represent duplicate aliquots of plasma membranes isolated from control ewes. [a, b] Means with different superscript letters differ ($p<0.05$).

EXPERIMENT 4. EFFECT OF PROGESTERONE ON PROGESTIN BINDING SITES

The effects of incubating plasma membranes with P_4 and P_4 + RU 486 on concentrations of P_4 binding sites are depicted in Fig. 10. While the binding of R5020 was similar between the control and the P_4 + RU 486-treated membranes, there was an increase in the concentration of progestin binding sites that occurred when membranes were preincubated with P_4 (control, 347 ± 64; P_4 + RU 486, 297 ± 74; P_4, 1529 ± 343 fmol/mg protein; $p<0.05$).

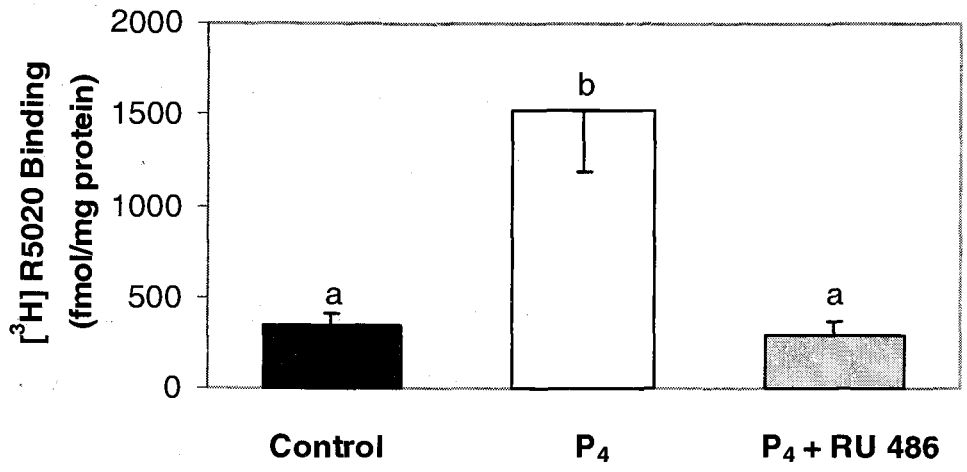

Figure 10. Binding of [^{3}H] R5020 to the endometrial OTR after *in vitro* exposure to P_4 or P_4 + RU 486. Means represent duplicate aliquots of endometrial plasma membranes isolated from samples collected from control animals (n = 4). $^{a, b}$Means (±SE) with different superscript letters differ ($p<0.05$).

DISCUSSION

Administration of P_4 to estrogen-primed OVX ewes in the present study suppressed the binding of OT to its endometrial receptor, which is similar to data reported by others (Sheldrick and Flint,1985; Vallet et al., 1990; Zhang et al., 1992). The ability of P_4 to interfere with the binding of OT was inhibited by treatment with the P_4 antagonist, RU 486. Mifepristone has been shown to block the action of P_4 by binding with high affinity to the intracellular P_4 receptor (Baulieu, 1989). These data offer further confirmation that, within the ovine system, P_4 exhibits a suppressive effect on OT binding to OT receptors and is thereby essential for the maintenance of uterine quiescence (Sheldrick and Flint, 1985; Vallet et al., 1990; Zhang et al., 1992; Stewart et al., 1993; Grazzini et al., 1998).

Gene transcription is known to be the primary mechanism for changes in OTR concentration within ovine endometrial cells (Hazzard et al., 1998; Wathes et al., 1996). Hazzard et al. (1998) demonstrated that P_4 has a suppressive effect on transcription of the OTR gene, thus resulting in OTR down-regulation. More recently, both Zingg et al. (1998) and Grazzini et al. (1998) demonstrated a suppressive effect of

physiologically relevant concentrations of P_4 on OT binding to OTR utilizing both rat uterine membranes and CHO cells stably transfected with the rat OTR expression vector. In our study, exposure of endometrial plasma membranes recovered from estrogen-primed OVX ewes to P_4 *in vitro* suppressed the binding of OT to its receptor. The suppressive effect of P_4 was reversed *in vitro* by plasma membrane incubation with the antagonist RU 486. The pattern of changes in bound OT among membranes exposed *in vitro* to P_4 and P_4 + RU 486 compared to that of controls were similar to those observed after *in vivo* treatment of ewes with these steroids. The data also demonstrates that freezing and thawing of the plasma membrane fractions were detrimental to OTR concentrations. Our data support the hypothesis that P_4 can act nongenomically to interfere with the binding of OT to its receptor in ovine endometrial plasma membrane. These data are also in agreement with those of Bogacki et al. (2002) who recently reported that P_4 acts at the level of the plasma membrane to interfere with OT binding to its receptor in the bovine endometrium.

In contrast to our data, Burger et al. (1999) reported the results of a study in which P_4 suppressed the binding of OT to OTR in CHO cells that were stably transfected with human OTR, but only by using a non-

physiological concentration of the steroid. Collectively, these data suggest that both the specific receptor and cell type are of primary consideration when evaluating the biological significance of P_4 and OT interaction. Because the membranous fraction utilized in our study was isolated from endometrial cells harvested from non-transgenic subjects, it would appear that within the ovine the suppressive effect of P_4 on the binding of OT to OTR may be a naturally occurring mechanism involving both genomic and nongenomic actions of the steroid.

In recent years there has been increasing experimental evidence for the existence of specific binding sites for P_4 in the plasma membrane of various cell types across species. Specific binding of P_4 has been demonstrated in ovarian follicular and luteal cell membranes of the bovine (Rae et al., 1998; Menzies et al., 1999), the plasma membrane of rat granulosa cells (Peluso et al., 2001), and the oocytes of *Xenopus laevis* (Godeau et al., 1978). Specific P_4 receptor binding sites within the plasma membrane of the corpus luteum of the human (Bramley and Menzies, 1988a), porcine (Bramley and Menzies, 1988b), ovine (Bramley and Menzies, 1994) and equine (Bramley et al., 1995) have also been reported.

It is presently unknown if P_4 could actually bind to the OT receptor or to an adjacent protein to exert a suppressive effect. The ability of P_4, R5020, RU 486, and OT to compete with the [^{3}H] R5020 suggests that progestin and OT may compete for the same or closely associated sites(Fig. 9). Data from Exp. 4 demonstrated that endometrial pre-incubation with P_4 resulted in a significantly greater number of progestin binding sites as compared to that of control and P_4 + RU 486-treated membranes. While there is some preliminary evidence that P_4 may bind to OT receptors, it is conceivable that P_4 may bind to a protein (other than the receptor) that ordinarily dimerizes with the receptor in response to the presence of OT. The OT receptor is a serpentine receptor, which traverses the plasma membrane seven times. There is experimental evidence for the dimerization of somatostatin (Rocheville et al., 2000), β_2-adrenergic (Hebert et al., 1996), GnRH (Conn et al., 1982), and δ opioid receptors (Cvejic and Devi, 1997), all of which are serpentine-G protein receptors. According to the proposed model (Fig. 11) each monomer of the receptor or associated protein contains a single binding site for P_4. In this scenario the exposure to P_4 would result in availability of a high affinity binding site for the steroid, which would prevent the dimerization of the OT receptor. Measurement of P_4 binding using an

exchange assay would thus result in data suggesting a doubling of the receptor sites on the membrane as occurred in Exp. 4. The data also suggest that the addition of the antagonist, RU 486, effectively blocked the ability of P_4 to bind to the OT receptor monomers, thus allowing the dimerization to occur.

In both the *in vivo* and *in vitro* studies, P_4 significantly suppressed the binding of OT to OT receptors, suggesting that P_4 acts both genomically and nongenomically to maintain uterine quiescence. The estimation of P_4 receptors (Exp. 3 and Exp. 4) provides complementary evidence for a natural "back up" system for the ovine reproductive system. The inhibitory action of P_4 is essential for the maintenance of pregnancy because its suppressive effects ultimately result in an inhibition of $PGF_{2\alpha}$ synthesis (Flint et al., 1990); an eicosanoid that plays a key role in initiating myometrial contractility. These data contribute to a foundation for evaluation of peptide-steroid "cross talk" at the level of the plasma membrane.

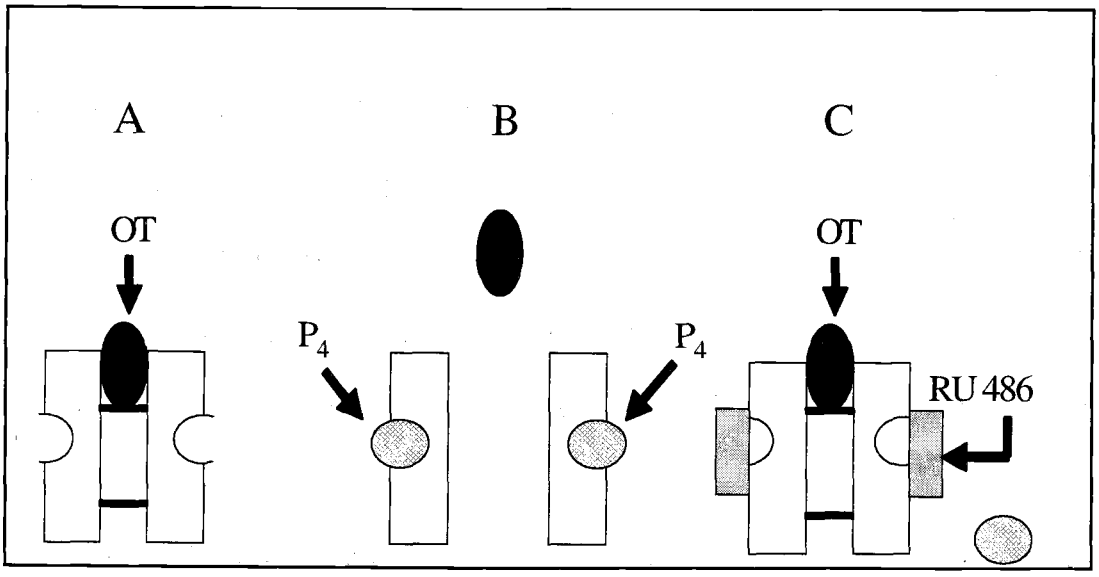

Figure 11. Proposed model depicting a mechanism by which progesterone may act at the level of the plasma membrane to interfere with binding of OT to its receptor in the ovine endometrium. (A) Binding of OT to OTR occurs following dimerization. (B) Individual OTR monomers are exposed to P_4 which binds to OTR monomers and inhibits dimerization thus preventing OT binding to OTR (C) The antagonist, RU 486, blocks the P_4 binding site on the OTR monomer thus enabling OTR dimerization and binding to OT as in (A).

GENERAL CONCLUSIONS

The role of progesterone in maintaining uterine quiescence by inhibiting the binding of oxytocin to its receptor is well established (Sheldrick and Flint 1985; Vallet et al., 1990, Zhang et al., 1992). The results of the *in vivo* study confirm the classically accepted inhibitory role of progesterone, as well as the antagonistic effect of RU 486. Within the ewe, progesterone inhibited the ability of oxytocin to bind to its receptor and mifepristone removed that block. The generation of similar results *in vitro* strengthens the case for a nongenomic interaction between progesterone and the oxytocin receptor. The isolation of the plasma membrane fraction prior to exposure of progesterone ensures that the results generated are due to non-nuclear mediated activities. *In vitro* progesterone exposure inhibited the binding of oxytocin to its receptor. That inhibition was reversed by concomitant membrane exposure to mifepristone. These data complement the research of both Zingg et al. (1998) and Grazzini et al. (1998), while providing an example of a nongenomic interaction in nontransfected cells. Also interesting to note is the detrimental effect of freezing and thawing on the oxytocin receptor

population, which provides valuable information regarding preferable handling techniques in future studies.

The ability of progesterone and promegestone to bind with relatively high affinities to the ovine endometrial plasma membrane fraction raises the question of a possible progestin binding site. It is well documented that progesterone does bind to putative receptors in the plasma membranes of a variety of cell types (Godeau et al., 1978; Rae et al., 1988; Menzies et al., 1999; Peluso et al., 2001). The results of the progestin binding study offer some insight into the possible site of progestin action upon the ovine endometrial plasma membrane. As expected, progesterone and promegestone effectively compete for the binding site. Also discovered was the fact that the RU 486 hindered the ability of the radiolabeled progestin to bind, which complements the research of Baulieu (1989) who demonstrated that RU 486 does bind to the intracellular progesterone receptor. Most importantly, data collected demonstrates that oxytocin was a potent inhibitor of progesterone binding. It appears that the site of progesterone binding may be the oxytocin receptor or a closely related protein.

In vitro membrane exposure to progesterone resulted in an increased number of progestin binding sites as compared to control or

RU 486-treated membranes. Plasma membrane preincubation with progesterone caused an increase in progestin binding and a decrease in oxytocin binding. This opposing relationship offers further support for the hypothesis that progesterone may be acting upon the oxytocin receptor or a closely associated protein.

The experimental evidence supporting the dimerization of serpentine G-protein-coupled receptors provides a foundation for the possible dimerization of the oxytocin receptor (Conn et al., 1982; Hebert et al., 1996; Cvejic and Devi, 1997; Rocheville et al., 2000). The data generated from the *in vivo* and *in vitro* analyses of oxytocin and progestin binding as well as the specificity of the progestin binding site complement the proposed model of oxytocin receptor dimerization (Fig. 11). This model is tenative and requires futher research for confimation.

These data add to the growing body of knowledge surrounding peptide-steroid "cross talk" at the level of the plasma membrane. More fully understanding the mechanisms by which hormones communicate to exert an effect has many far-reaching implications.

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