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In Vivo and In Vitro Responses of the Bovine Corpus Luteum After Exposure to Exogenous Gonadotropin-Releasing Hormone and Prostaglandin $F_{2\alpha}$

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Administration of gonadotropin-releasing hormone (GnRH) early in the estrous cycle has been shown to cause subsequent altered luteal function. To determine whether membrane-related events may be involved in GnRH-attenuated luteal function, corpora lutea (CL) were removed from beef heifers on day seven of the estrous cycle after iv injection of GnRH or saline on day two of the cycle (n = 5/group). Luteal slices were incubated with saline (control), luteinizing hormone (LH), or 8-bromo-cAMP for 2 h. In vivo administration of GnRH reduced LH and cAMP-stimulated progesterone production by tissue ($p < 0.01$), but basal progesterone production was not affected ($p > 0.05$). Luteal adenylyl cyclase activity did not differ between saline and GnRH-treated animals ($p > 0.05$). Then, to examine if early administration of GnRH alters response of the CL to prostaglandin (PG) $F_{2\alpha}$, beef heifers were injected with GnRH as described above (n = 4/group), and then injected with $PGF_{2\alpha}$ on day eight and the CL removed 60 min later. Blood samples were collected for oxytocin (OT) analysis at frequent intervals after $PGF_{2\alpha}$ injection and for progesterone at 0 and 60 min. Induction of the early response gene c-*jun* or release of OT by $PGF_{2\alpha}$ was not altered by GnRH injection ($p > 0.05$). Injection of $PGF_{2\alpha}$ decreased serum progesterone by 60 min postinjection ($p < 0.05$), but concentrations of this steroid were unaffected by GnRH ($p > 0.05$). Collectively, these data suggest that GnRH-induced alteration of bovine luteal function may be owing to events distal to cAMP synthesis that do not interfere with $PGF_{2\alpha}$-induced expression of c-*jun* or OT release, cellular phenomena involved in luteolysis.

Key Words: Corpus luteum; GnRH; $PGF_{2\alpha}$; c-*jun*; oxytocin; progesterone.

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Introduction

Ford and Stormshak (1978) first observed that injection of gonadotropin-releasing hormone (GnRH) into cows during metestrus reduced serum concentrations of progesterone later in the estrous cycle. This effect was confirmed by Rodger and Stormshak (1986), who found that GnRH injected on day two of the estrous cycle reduced serum progesterone levels beginning on day eight of the cycle. Other studies have reported similar results in both the cow and the ewe (Lucy and Stevenson, 1986; Slayden and Stormshak, 1990), although some have found that injection of GnRH actually increases serum concentrations of progesterone later in the cycle (Mee et al., 1993).

The mechanisms of action of any GnRH-induced alteration of luteal function remain unresolved. Effect of GnRH on the corpus luteum (CL) appears to be indirect, at least in domestic animals, because no GnRH receptors have been detected in CL or follicles of cows, ewes, or sows (Brown and Reeves, 1983). Slayden and Stormshak (1990) found that injection of luteinizing hormone (LH) into ewes could mimic the effect of GnRH injection on serum progesterone levels later in the cycle, thus supporting the concept that the effects of exogenous GnRH in ruminants are indirect. Injection of GnRH on day two reduced the number of luteal LH receptors on days 8 and 14 of the cycle, suggesting that the effect of GnRH may be mediated by an increased number of large luteal cells (Rodger and Stormshak, 1986). Large luteal cells contain few LH receptors and produce high basal amounts of progesterone, whereas small luteal cells have many LH receptors and produce increased quantities of progesterone in response to LH (Ursely and Leymarie, 1979; Koos and Hansel, 1981; Fitz et al., 1982). Mee et al. (1993) confirmed the hypothesis by finding an increased large luteal cell to small luteal cell ratio in CL of cows that had received GnRH 12 h after the onset of estrus. That study did not, however, rule out potential alteration in luteal function at the cellular level.

Few studies have examined responses of the CL to injection of prostaglandin (PG) $F_{2\alpha}$ after previous administration of GnRH. Release of oxytocin in response to $PGF_{2\alpha}$

is an indicator that the CL is undergoing luteolysis (McCracken and Schramm, 1983). Large, but not small luteal cells appear to express high-affinity receptors for $PGF_{2\alpha}$ on their cell surfaces (Fitz et al., 1982; Gadsby et al., 1990). Oxytocin (OT) is produced by large luteal cells, stored in secretory granules and secreted by exocytosis through $PGF_{2\alpha}$-induced activation of protein kinase C (PKC; reviewed by Stormshak et al., 1995). Because injection of GnRH early in the cycle is believed to cause an increased large luteal cell to small luteal cell ratio (Mee et al., 1993), it is possible that CL from GnRH-treated cows would respond to the luteolytic stimulus of $PGF_{2\alpha}$ with increased secretion of oxytocin. Additionally, it has been suggested that the early response gene c-*jun* and its product may play a role in luteolysis (Khan et al., 1993, 1994), as well as apoptosis in some cell types (Estus et al., 1994; Goldstone and Lavin, 1994). Apoptosis has been implicated as one potential mechanism of bovine luteal regression (Juengel et al., 1993; Zheng et al., 1994). However, studies specifically designed to examine the effect of GnRH injection early in the estrous cycle on $PGF_{2\alpha}$-induced luteolysis in midcycle have not been conducted.

In the present study, two experiments were conducted to examine further the action of exogenous GnRH on bovine luteal cell function. More specifically, the first experiment was conducted to determine whether plasma membrane-related events, such as binding of LH to its receptor, stimulation of adenylyl cyclase, and accumulation of cAMP, contribute to GnRH-altered luteal function. In the second experiment, we sought to determine whether c-*jun* message can be upregulated in the bovine CL in response to $PGF_{2\alpha}$ on day eight of the estrous cycle, and if so, whether this $PGF_{2\alpha}$-induced effect could be altered by administration of GnRH on day two of the cycle. A corollary objective of this latter experiment was to determine if early administration of GnRH altered systemic concentrations of OT and progesterone during $PGF_{2\alpha}$-induced luteolysis.

Results

Effects of GnRH Injection on In Vitro Progesterone Production

Slices of CL removed from heifers on day seven of the estrous cycle responded to LH and 8-bromo-cAMP with increased progesterone production compared with incubated controls ($p = 0.0001$). However, injection of GnRH on day two significantly reduced progesterone production by luteal slices in response to LH ($p < 0.01$) and cAMP ($p < 0.001$) but did not affect basal progesterone production by the tissue slices ($p > 0.05$; Fig. 1). Neither nonactivated adenylyl cyclase activity nor that activated with GTPγS was affected by in vivo injection with GnRH ($p > 0.05$; Fig. 2).

Effects of GnRH and $PGF_{2\alpha}$ on the CL In Vivo

Administration of $PGF_{2\alpha}$ to beef heifers on day eight of the estrous cycle increased luteal expression of the early

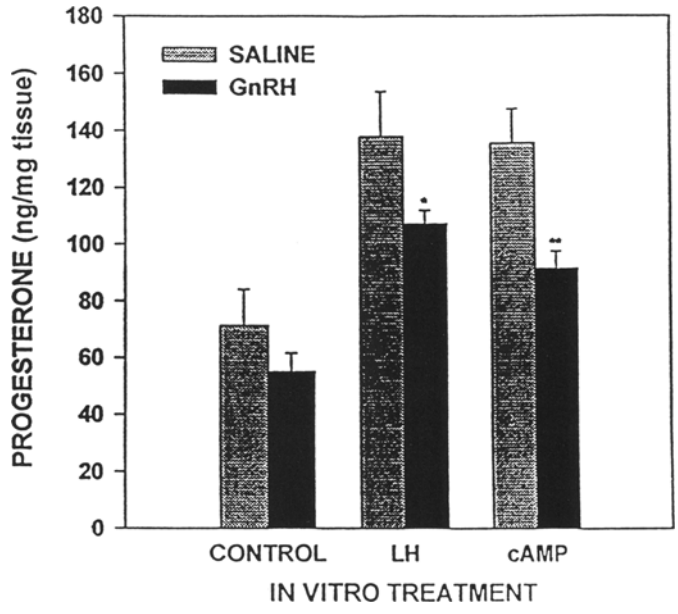

Fig. 1. In vitro progesterone production by day seven luteal slices from beef heifers injected with saline or GnRH on day two of the estrous cycle. In vitro treatments of LH (50 ng/mL) and 8-bromo-cAMP (15 m*M*) stimulated progesterone production ($p = 0.0001$). In vivo administration of GnRH reduced progesterone production in response to LH (*, $p < 0.01$) and cAMP (**, $p < 0.001$) treated slices, but not control slices ($p > 0.05$).

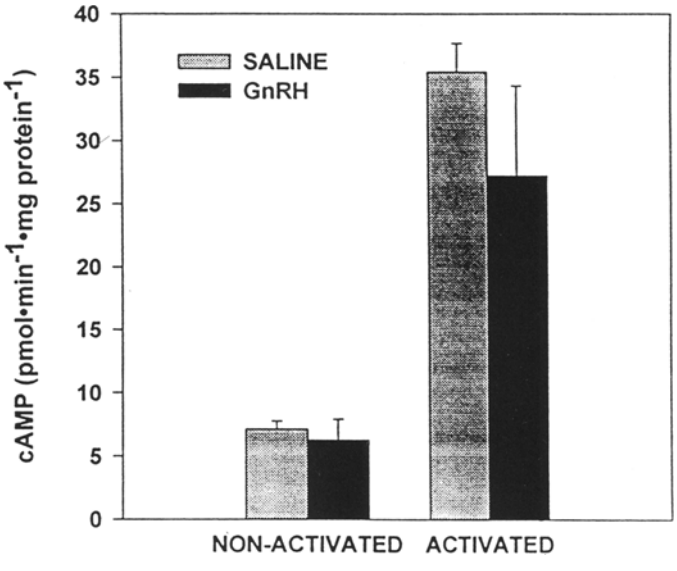

Fig. 2. Adenylyl cyclase activity in CL of beef heifers injected with saline or GnRH expressed as pmol cAMP formed/min/mg protein. The enzyme assay was performed without (nonactivated) or with (activated) the addition of 0.1 m*M* GTPγS. Administration of GnRH was without effect on adenylyl cyclase activity ($p > 0.05$).

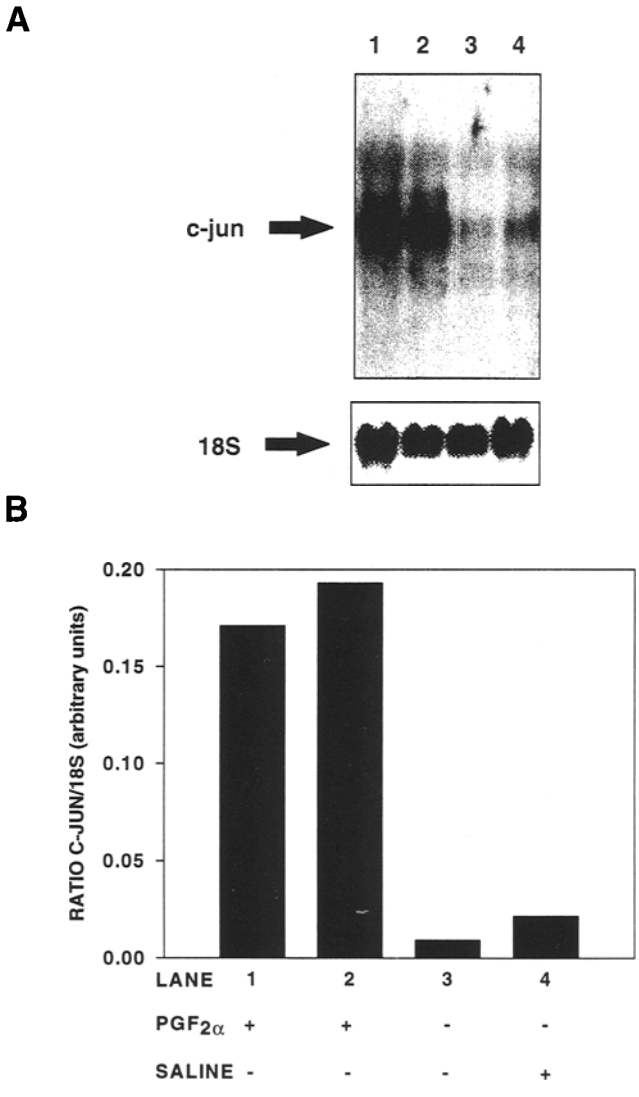

Fig. 3. Northern blot of c-*jun* mRNA (2.7 kb) from day eight bovine CL **(A)** and density of c-*jun* mRNA signal normalized to the 18S signal for each lane **(B)**. Lanes 1 and 2: animals received an injection of 500 μg cloprostenol ($PGF_{2\alpha}$) 60 min prior to removal of CL. Lane 3: animal received no injection. Lane 4: animal received injection of saline 60 min prior to removal of CL. 18S rRNA was probed to determine equality of loading. Quantitation was performed using volume quantitation of equal areas for a specific signal. Background was corrected for by subtraction of the local average around the area quantitated. The legend below the graph indicates the treatment received by individual beef heifers 60 min prior to CL removal. A minor transcript at 3.6 kb was detected immediately above the 2.7 kb major band in lanes 1 and 2.

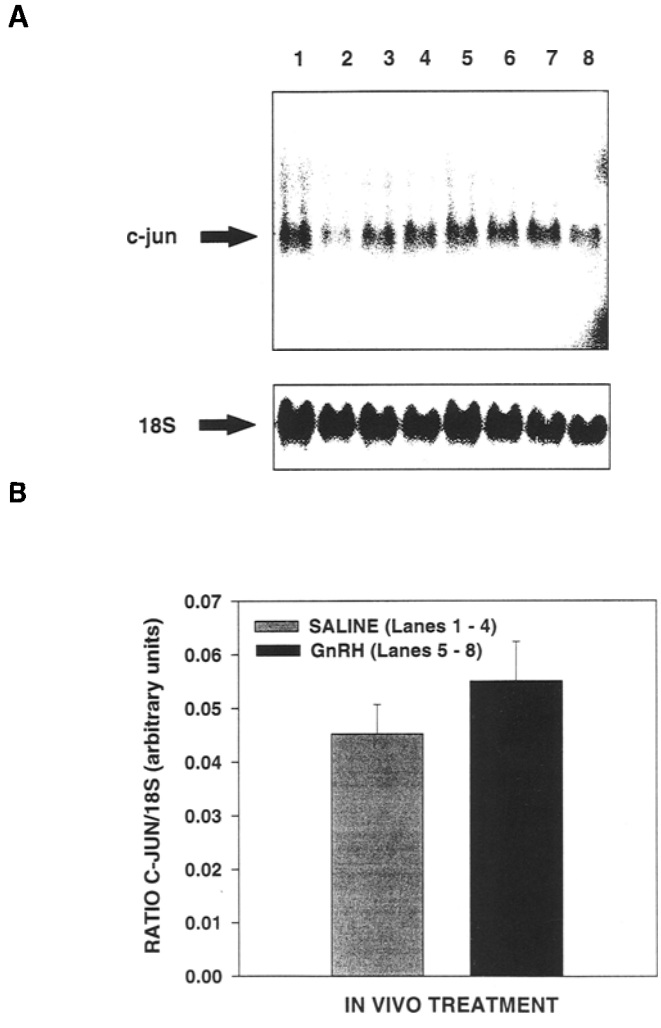

Fig. 4. Northern blot of c-*jun* mRNA from day eight bovine CL 60 min after injection of $PGF_{2\alpha}$ **(A)** and mean (±SE) densities of c-*jun* mRNA signal normalized to the 18S signal **(B)**. Animals had received an injection of saline (lanes 1–4) or GnRH (lanes 5–8) on day two of the estrous cycle. All animals received an injection of $PGF_{2\alpha}$ 60 min prior to CL removal. 18S rRNA was probed to determine equality of loading. There was no alteration in the $PGF_{2\alpha}$-induced c-*jun* expression by early administration of GnRH.

response gene c-*jun* at 60 min after injection when compared with control animals (Fig. 3A). A major transcript was found at 2.7 kb, and a minor transcript was found at approx 3.6 kb (Fig. 3A, lanes 1 and 2). When the amount of major transcript was quantitated and expressed relative to signal intensity for 18S rRNA, treated animals showed a 10- to 20-fold higher induction of c-*jun* than control animals (Fig. 3B). However, GnRH administration on day two was not able to alter this $PGF_{2\alpha}$-induced expression of the 2.7-kb c-*jun* transcript at 60 min after injection, either positively or negatively (Fig. 4A) when normalized to signal for 18S rRNA (Fig. 4B, $p > 0.05$). Plasma OT increased significantly after $PGF_{2\alpha}$ injection ($p < 0.001$), but there were no differences between saline and GnRH-treated animals (Fig. 5). The mean 30- and 35-min plasma OT samples were not significantly different from the time 0 sample ($p > 0.05$), indicating that the OT levels had returned to baseline by 30 min after $PGF_{2\alpha}$ injection. Sample means at all other times were significantly different from the time 0 mean ($p < 0.01$). Analysis of peak plasma concentrations of OT (pg/mL) for individual animals in the saline vs GnRH treatments revealed no differ-

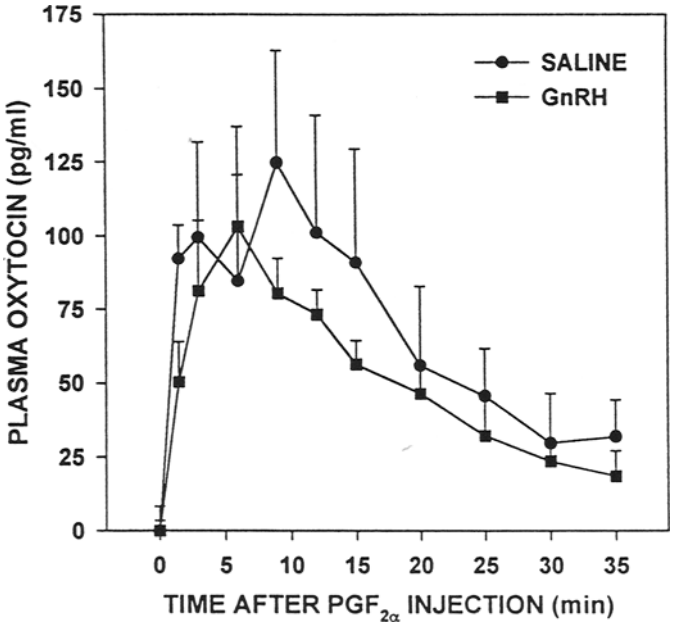

Fig. 5. Plasma OT concentrations in beef heifers on day eight of the estrous cycle following $PGF_{2\alpha}$ injection at time 0. "Saline" and "GnRH" indicate treatments administered on day two of the estrous cycle (n = 4 animals/group).

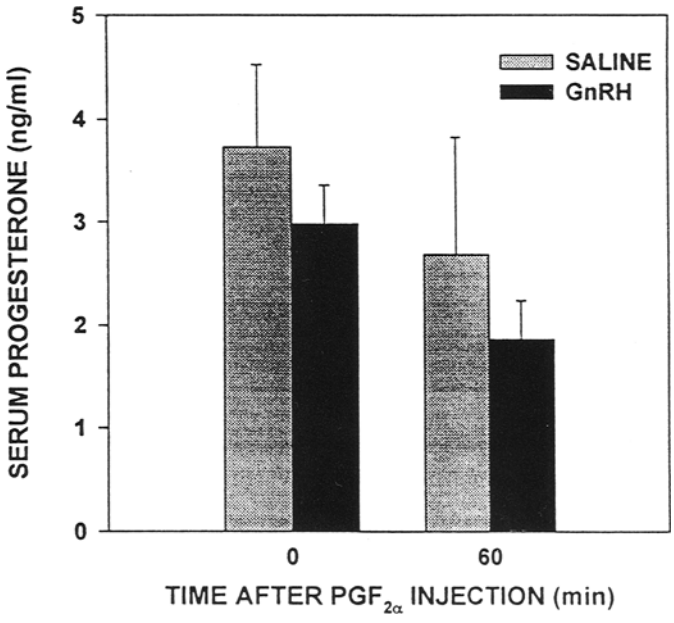

Fig. 6. Serum concentrations of progesterone at 0 and 60 min after $PGF_{2\alpha}$ injection into beef heifers on day eight of the estrous cycle. There was no effect of GnRH injection. However, $PGF_{2\alpha}$ injection significantly decreased progesterone concentrations in both saline- and GnRH-treated animals by 60 min after injection ($p < 0.05$).

ence between injections (saline, 129.1 ± 36.5; GnRH, 124.5 ± 23.5; $p > 0.05$). Peak OT concentrations for all animals occurred between 3 and 12 min after $PGF_{2\alpha}$ injection. Oxytocin in luteal tissue (ng/g tissue) removed 60 min after $PGF_{2\alpha}$ also did not change owing to GnRH treatment (saline, 24.07 ± 8.6; GnRH, 19.31 ± 6.0; $p > 0.05$). Serum concentrations of progesterone (ng/mL) significantly declined by 60 min after $PGF_{2\alpha}$, compared with the zero time sample in both groups (saline 0 min, 3.73 ± 0.79; 60 min, 2.69 ± 1.1; GnRH 0 min, 2.98 ± 0.38; 60 min, 1.87 ± 0.38; $p < 0.05$; Fig. 6), but GnRH injection did not alter progesterone concentrations ($p > 0.05$). However, there was a trend for serum concentrations of progesterone to be lower in GnRH-treated than in control heifers.

Discussion

Results of the present experiment suggest two potential mechanisms that may explain the alteration in luteal function found after GnRH injection. One possibility is that cellular components responsible for progesterone production by normal LH stimulation are functionally altered. This could explain why progesterone production was reduced in GnRH-exposed luteal tissue in response to both LH and cAMP. If this is true, then this functional difference must occur at a point distal to the accumulation of cAMP in the small luteal cell. In this experiment, stimulation of progesterone with cAMP was depressed in CL from GnRH-treated heifers compared with controls. In addition, the response of this tissue to LH was similar to the response to cAMP. The finding that there is no difference in luteal adenylyl cyclase activity between saline- and GnRH-treated animals further supports this premise, because adenylyl cyclase is the enzyme responsible for the conversion of ATP to cAMP. Thus, the adenylyl cyclase/cAMP system was not able to overcome the GnRH-induced inhibition of progesterone production by CL in response to LH in vitro.

This idea is consistent with results from Rusbridge et al. (1993), who performed an experiment using dissociated luteal cells from untreated heifers on day seven of the cycle, or heifers injected with GnRH on day six followed by CL removal on day 13 of the estrous cycle. Their results of stimulation of the cells with LH and dibutyryl cAMP led them to conclude that alteration in steroidogenic response of GnRH-exposed CL occurs at least at a point distal to the LH receptor. There are a number of other steps in the biochemical pathway leading to production of progesterone by the luteal cell that could potentially be affected subsequent to GnRH injection early in the cycle (Leers-Sucheta and Stormshak, 1991).

A second possibility is that no functional difference in luteal cell types exists between animals that have received an injection of GnRH or saline. Change in cellular composition of the corpus luteum has been proposed as the mechanism by which alteration of luteal function occurs. Mee et al. (1993) found that GnRH administered 12 h after the onset of $PGF_{2\alpha}$-induced estrus (at the time of artificial insemination) in repeat-

breeder dairy cows increased the large-to-small luteal cell ratio in CL from these animals on day 10 after estrus. Saline-injected control animals had a ratio of 14% large to 86% small cells, whereas those cows that received GnRH had a ratio of 31% large to 69% small cells. These researchers conducted an in vitro experiment as well, and found that early administration of GnRH did not affect basal progesterone production by day 10 luteal tissue slices, but reduced LH-stimulated progesterone production. These data are similar to those from the first experiment of our study. Hence, the results of our study may simply reflect responses of a reduced population of small luteal cells in GnRH-treated animals. However, if an increased large luteal cell to small luteal cell ratio is the only explanation for the findings by Mee et al. (1993) and the results of the present study, then it may be presumed that significantly higher basal progesterone production by GnRH-exposed CL should occur, rather than just a reduction in LH (and cAMP)-stimulated progesterone production. However, this was not the case in either study.

Davis et al. (1995) have shown that $PGF_{2\alpha}$-stimulated PKC activates the MAP kinase cascade in causing the release of transforming growth factor β (TGFβ) from bovine luteal cells. They hypothesized that this cascade causes phosphorylation of transcription factors in the luteal cell, presumably including c-*jun*. The induction of c-*jun* in the CL by $PGF_{2\alpha}$ found in this investigation is believed to be the first report of this phenomenon with the exception of the rat (Khan et al., 1993, 1994). It should be noted that c-*jun* and other transcription factors, such as c-*fos* and c-*myc*, have also been implicated in events, such as follicular growth and atresia, as well as corpus luteum maintenance and regression (Khan et al., 1994; Li et al., 1994; Peluso et al., 1995). The present study has shown a message for c-*jun* at 2.7 (major transcript) and 3.6 kb (minor transcript) in the bovine corpus luteum. The size of the transcripts is consistent with those reported for other steroidogenic tissues. In the rat CL, a major transcript at 2.6 kb and a minor transcript at 3.5 kb have been found (Khan et al., 1993). In bovine adrenal tissue, the major transcript is found at 2.7 kb and a minor transcript at 3.4 kb (Clark et al., 1992). Additionally, mouse Y1 adrenocortical cells contain transcripts for c-*jun* of 2.7 and 3.6 kb (Kimura et al., 1993).

Early administration of GnRH, however, did not alter $PGF_{2\alpha}$-induced expression of the major c-*jun* transcript in the bovine CL in this experiment. There are several possible explanations for this occurrence. One is simply that any effect exogenous GnRH may have on the CL did not affect those pathways that involve expression of this early response gene in luteal cells. Additionally, expression of c-*jun* at the time-point after $PGF_{2\alpha}$ injection (60 min postinjection) may not have been altered, but it is possible that the rise in c-*jun* message or its decline to baseline quantities was altered. Because the study was not a time-course experiment, changes in the rate of c-*jun* induction, rather than in the amount of induction alone, cannot be ascertained at the present time. It is also possible that c-*jun* is differentially up- or downregulated in large or small luteal cells, a process that could be affected by GnRH, but not detected in whole CL extract.

Release of OT from the bovine CL after injection of $PGF_{2\alpha}$ can be used as another indicator of luteolytic potential of GnRH-exposed CL. In the present study, however, concentrations of neither plasma OT nor OT in luteal tissue 60 min after injection of $PGF_{2\alpha}$ were significantly altered by early administration of GnRH. In contrast, Whitmore (1995) found a reduction in naturally secreted luteal OT on days 12 and 14 in intact ewes that had been injected with GnRH on days two and three of the estrous cycle. In the present study, serum concentrations of progesterone did decline by 60 min after injection of $PGF_{2\alpha}$, but at neither 0 nor 60 min after $PGF_{2\alpha}$ were the serum concentrations of progesterone significantly affected by early administration of GnRH. This result is not necessarily surprising, since previous studies have shown variable serum progesterone responses after early administration of GnRH (Stevenson et al., 1984; Lucy and Stevenson, 1986; Rodger and Stormshak, 1986; Mee et al., 1993). Nevertheless, it should be noted that serum concentrations of progesterone in GnRH-treated heifers were lower than those of controls. Additionally, Macmillan et al. (1985) also found that plasma concentrations of progesterone declined similarly in cows pretreated with the GnRH agonist Buserelin and control animals after injection of $PGF_{2\alpha}$. Interestingly, injection of the GnRH analog at various times during diestrus, 15 min, 24 h, or 72 h before injection of $PGF_{2\alpha}$ did not alter functional luteolysis, but prevented or slowed structural luteolysis. In the present study, alteration of structural changes could not be assessed because the CL were removed 60 min after injection of $PGF_{2\alpha}$.

In summary, early administration of GnRH inhibited LH and cAMP-stimulated progesterone production from day seven bovine luteal slices, but did not alter luteal adenylyl cyclase activity. The results may be explained in a manner consistent with the hypothesis that an increased large luteal cell to small luteal cell ratio exists after GnRH injection. However, the possibility of functional differences in cells of corpora lutea exposed to exogenous GnRH cannot be fully excluded. Both c-*jun* gene expression and plasma concentrations of oxytocin increased following injection of $PGF_{2\alpha}$ into beef heifers on day eight of the estrous cycle, whereas serum concentrations of progesterone declined. These are expected responses of the CL when exposed to a luteolytic dose of $PGF_{2\alpha}$. However, injection of GnRH on day two of the cycle was unable to alter these $PGF_{2\alpha}$-induced events. Alteration of luteal function after early administration of GnRH does not appear to involve these $PGF_{2\alpha}$-responsive cellular mechanisms.

Materials and Methods

Animals and Experimental Design

An experiment was conducted to determine if luteal cell membrane-related events contribute to attenuated progesterone production by CL of GnRH-treated heifers. All experimental procedures were approved by the Institutional Animal Care and Use Committee. Beef heifers of mixed breeds were checked twice daily for estrus using a vasectomized bull. On day two of the estrous cycle (estrus = day zero of the cycle), animals were injected iv with 2 mL sterile saline (0.9% NaCl) or GnRH (Cystorelin, 50 μg/mL, Sanofi Animal Health, Overland Park, KS; n = 5 animals/group). On day seven, animals were restrained for surgery to remove the CL. Caudal epidural anesthesia was induced by injection of 4 mL lidocaine hydrochloride (2%) into the coccygeal spinal column and the CL removed *per vaginum*. Corpora lutea were transported to the laboratory in sterile, phenol red-free Ham's F-12 medium (Nutrient Mixture F-12 [Ham], Gibco Laboratories, Grand Island, NY) containing 14 m*M* sodium bicarbonate, 24 m*M* HEPES, and 30 μg/mL gentamicin (Gibco), pH 7.3. For determination of adenylyl cyclase activity, approx 100 mg of tissue were homogenized in 2 mL sucrose buffer (27% sucrose [w/w], 1 m*M* EDTA, 10 m*M* Tris, pH 7.5), immediately frozen in liquid nitrogen, and stored at –80°C until the enzyme activity assay was performed.

The remainder of the CL was sliced to 0.3-mm thickness, washed three times in medium, and aliquoted to eight 10-mL Erlenmeyer flasks (approx 100 mg/flask), each containing 2 mL incubation medium. Incubation medium consisted of Ham's F-12 (as described above) plus 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL selenium (ITS, Sigma, St. Louis, MO). Treatments were then added to each flask: 20 μL saline were added to four flasks (two unincubated controls, two incubated controls), two flasks received bovine LH dissolved in 20 μL saline (final concentration: 50 ng/mL; USDA-bLH-B-6, Doug Bolt, USDA Animal Hormone Program, Beltsville, MD), and 8-bromo-cAMP (Sigma) was added to two flasks at a final concentration of 15 m*M*. Flasks were gassed with 95% O_2–5% CO_2 for several seconds each and capped with silicone stoppers. A total of 4 mL of cold ethanol was then immediately added to the two unincubated control flasks to preclude further progesterone synthesis. The remaining flasks were incubated for 2 h at 37°C in a Dubnoff shaking water bath. After 2 h, cold ethanol was added to these flasks to terminate the incubation. Tissue plus medium samples were stored at –20°C until extraction and determination of progesterone content by radioimmunoassay.

For the second phase of the investigation, a preliminary experiment was first performed to determine if $PGF_{2\alpha}$ altered c-*jun* expression in the bovine CL as has been described for the rat (Khan et al., 1993, 1994). Two beef heifers were injected with 500 μg cloprostenol, a $PGF_{2\alpha}$ analog (Estrumate, Mobay Corp., Shawnee, KS) iv on day eight of the estrous cycle; one heifer received an injection of saline. Corpora lutea were removed 60 min after injection, frozen in liquid nitrogen, and stored at –80°C until RNA analysis. An additional day-eight CL from a heifer that did not receive an injection also served as a control.

After the induction of c-*jun* by $PGF_{2\alpha}$ was confirmed, beef heifers were injected with saline or GnRH on day two of the estrous cycle (n = 4 animals/group). On day eight of the cycle, animals were restrained and the jugular vein catheterized with a 16-gage, 8.3-cm Angiocath catheter (Deseret Medical Inc., Becton Dickinson and Co., Sandy, UT). Clotting in the catheter was prevented by infusion of a 3.5% sodium citrate-0.2% oxytetracycline solution. Blood samples were then collected with Vacutainer tubes (Becton Dickinson, Rutherford, NJ) for determination of serum progesterone or plasma OT (collected in heparinized Vacutainers), and designated "time zero" samples. Ten microliters of 5 mg/mL 1,10-phenanthroline (Sigma) and 20 μL of 0.5*M* EDTA were added immediately to this and all subsequent OT samples to prevent oxytocinase activity (Kumarasen et al., 1974). All blood samples were placed on ice after collection. Next, 500 μg cloprostenol ($PGF_{2\alpha}$) were injected via the catheter (time zero). Blood samples for OT were collected as above at 1.5, 3, 6, 9, 12, 15, 20, 25, 30, and 35 min after $PGF_{2\alpha}$ injection. At 60 min after injection, a blood sample was taken for determination of progesterone, and the CL removed as described above and frozen in liquid nitrogen. Corpora lutea were stored at –80°C until analysis for c-*jun* expression and tissue concentrations of OT. Blood samples were centrifuged for 10–15 min at 2540*g* at 4°C. Serum samples were allowed to clot at 4°C overnight before centrifugation. Both plasma and serum were stored at –20°C until OT or progesterone radioimmunoassay was performed.

Progesterone Radioimmunoassay

Tissue plus medium samples were extracted by the procedure of Koligian and Stormshak (1976). Each sample was homogenized in ethanol after addition of [^{3}H]progesterone (Dupont NEN, Boston, MA) to the sample for determination of extraction efficiency. Samples were filtered into flasks through Whatman No. 1 filter paper with a total of 18 mL ethanol. Flasks were roto-evaporated at 45°C until samples were nearly dry, resuspended in 3 mL distilled water, and extracted with 20 mL benzene:hexane (1:2). The mean extraction efficiency for tissue plus medium samples from the first experiment was 68.7 ± 0.8%. Each sample was corrected for recovery using its own extraction efficiency. For the second experiment, progesterone in duplicate serum samples (100 μL each) was extracted with 2 mL benzene:hexane (1:2) by vortexing for 30 s. A third tube for each sample containing 100 μL serum plus [^{3}H]progesterone was extracted for determination of extraction efficiency. Mean extraction efficiency, used to correct all samples for recovery, was 85.5%. Radioimmu-

noassay was performed using antiprogesterone-11-BSA (Gordon Niswender, Colorado State University, Fort Collins, CO) in a 1:3500 dilution. Intra-assay and interassay coefficients of variation were determined using a progesterone sample of 75 pg/100 μL ethanol (18 tubes/assay), and for the first experiment were 10.1 ± 0.5 and 3.8%, respectively (n = 7 assays). For the second experiment, intra-assay coefficient of variation was 7.34% (n = 1 assay).

Adenylyl Cyclase Activity Assay

Adenylyl cyclase activity was measured in luteal homogenates using reagents and sample preparation described by Agudo et al. (1984) and column chromatography for separation of cAMP according to Birnbaumer et al. (1988). All reagents were purchased from Sigma, unless otherwise noted. Reaction mixtures in a final volume of 100 μL consisted of 5 m*M* $MgSO_4$, 50 m*M* HEPES, 0.2% BSA (w/v), 1 m*M* cAMP, 10,000 cpm [^{3}H]cAMP (Dupont NEN), 4 m*M* creatine phosphate, 2.5 U creatine phosphokinase (Calbiochem, La Jolla, CA), 0.1 m*M* ATP containing approx 1 × 10^6 cpm [α-^{32}P]ATP (DuPont NEN), and 50 μL luteal homogenate. Adenylyl cyclase activity was determined in both the presence (activated) and absence (nonactivated) of 0.1 m*M* GTPγS, each in duplicate for each luteal sample. A reaction mixture without luteal homogenate was incubated with each set of samples as a reaction blank to determine nonspecific incorporation. Reaction mixtures were incubated for 10 min at 30°C, and the reaction stopped with 1 mL 0.17*N* perchloric acid. Samples were passed through two columns containing AG 50W-4X, 200–400 mesh, H^+ form resin (Bio- Rad Laboratories, Hercules, CA) and alumina oxide. Cyclic AMP was eluted by addition of 4 mL 0.1*M* imidazole HCl buffer. [^{3}H]cAMP in eluted samples and in the original reaction mixture were used to determine percentage recovery. [^{32}P]cAMP in the samples was used to determine conversion from [^{32}P]ATP with [^{32}P] in the reaction blank subtracted from each sample. Protein concentration in each luteal homogenate was determined using the Bio-Rad protein assay, and adenylyl cyclase activity was expressed as cAMP formed in pmol/min/mg protein.

OT Radioimmunoassay

Oxytocin in plasma samples was extracted and assayed as recently described by Orwig et al. (1994) by methods adapted from Abdelgadir et al. (1987) and Schams (1983). Oxytocin was extracted from tissue samples by the method of Tsang et al. (1990) before analysis. For the plasma extraction, a Waters vacuum manifold was utilized with Sep-Pak Plus C-18 cartridges (Waters Chromatography Division, Millipore, Milford, MA). Mean extraction efficiency for plasma was 75.0 ± 0.7% and for tissue 93.6 ± 1.6%. All sample values were adjusted for recovery. Oxytocin antibody was generously provided by Dieter Schams, Technical University of Munich, Freising-Weihenstephan, Germany. Intra- and interassay coefficients of variation were 9.6 ± 0.7 and 3.4%, respectively (n = 5 assays).

RNA Extraction and Northern Blotting

For extraction of RNA, luteal tissue (approx 300 mg) was pulverized with a mortar and pestle under liquid nitrogen and then placed in 10 vol (3 mL) TRIzol reagent (Gibco), a monophasic solution of phenol and guanidine isothiocyanate (Chomczynski, 1993). Extraction was performed according to the manufacturer's protocol. Recovered RNA was dissolved in 50 μL diethylpyrocarbonate (DEPC)-treated water. Quantity and purity of RNA were determined spectrophotometrically.

To denature the RNA, samples were heated to 55°C for 15 min with sample preparation buffer (1 part 10 × MOPS buffer [0.2*M* MOPS, 80 m*M* sodium acetate, pH 7.0], 1.75 parts deionized formaldehyde, and 5 parts deionized formamide) in a ratio of 9:31. Denatured samples plus 2 μL gel-loading buffer (50% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF) were loaded onto a formaldehyde agarose gel (1%) containing approx 0.8 μg/mL ethidium bromide. Electrophoresis was performed overnight at 30 V in 1X MOPS buffer. The gel was then rinsed for 1 h in DEPC-treated water and then soaked in 6X SSC buffer for 10–15 min (1X SSC = 0.15*M* sodium chloride, 15 m*M* sodium citrate, pH 7.0). RNA was transferred onto a Nytran Plus nylon membrane (Schleicher and Schuell, Keene, NH) by capillary transfer. After 48 h, the membrane was rinsed briefly with 5X SSC and then UV crosslinked at 120,000 μJ/cm^2.

The membrane was prehybridized at 42°C for at least 6 h with 200 μL/cm^2 prehybridization solution (50% deionized formamide, 5X SSC, 50 m*M* K_2PO_4, pH 8.0, 5X Denhart's solution [0.1% each BSA, Ficoll and polyvinylpyrrolidone; Sigma], 100 μg/mL salmon testes DNA for hybridization [Sigma], and 0.1% SDS) and then hybridized with the appropriate labeled cDNA probe. The probe for c-*jun* was made from a plasmid insert of the full-length mouse c-*jun* cDNA obtained from Rodrigo Bravo, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ. Probes made from this template have been successfully used to examine c-*jun* expression in bovine tissue (Clark et al., 1992). The probe for 18S ribosomal RNA was made from a plasmid insert containing 80 bp of the human 18S rRNA gene (Ambion, Austin, TX). Probes were made from the DNA templates by random hexanucleotide priming with [^{32}P]dCTP (Dupont NEN) as the radioactive label (Prime-a-Gene Labelling System, Promega, Madison, WI). Unincorporated label was removed by passage though a Sephadex G-50 column (Quick Spin columns, Boehringer Mannheim, Indianapolis, IN).

Labeled probe was added to the hybridization solution to obtain approximately 1.5–3 × 10^6 cpm/mL. Hybridization solution (50 μL/cm^2 membrane) contained 50% deionized formamide, 5X SSC, 20 m*M* K_2PO_4, pH 6.5, 1X Denhart's solution, 100 μg/mL salmon testes DNA for hybridization, and 0.1% SDS. After overnight hybridization (approx 16 h)

at 42°C, membranes were washed in 2X SSC, 0.1% SDS for 30 min at room temperature, and then washed for 15 min twice at 50°C. Some were washed further with 0.1X SSC and 0.1% SDS at 50°C for 30 min. Blots were exposed to a storage phosphor screen (Molecular Dynamics, Sunnyvale, CA) overnight or for several days. Screens were scanned by a PhosphorImager SI and visualized with ImageQuaNT software (Molecular Dynamics). Between probing for c-*jun* and 18S rRNA, membranes were stripped with 50% formamide, 6X SSPE (1X SSPE = 0.18*M* NaCl, 10 m*M* sodium phosphate, 1 m*M* EDTA, pH 7.4) for 30–45 min at 65°C.

Signal densities were quantitated with ImageQuaNT using volume quantitation of equal areas for any given signal. Background correction was computed by the local average method. Intensity of the c-*jun* signal relative to the 18S signal was calculated to assure that comparisons were made between equal quantities of RNA.

Statistical Analysis

Data on tissue plus medium concentrations of progesterone were analyzed by ANOVA for an experiment of split-plot design using the general linear model procedures of SAS (1993). Treatment duplicates were averaged, and the mean values used for statistical analysis after subtraction of each sample's corresponding mean unincubated control value. For the second experiment, effects of treatments on both plasma OT and serum progesterone were analyzed by repeated measures ANOVA using the general linear model procedures of SAS. Differences among tissue plus medium progesterone and mean plasma concentrations of OT at sampling times after $PGF_{2\alpha}$ injection were tested by the Least-Significant Difference test. Significant differences among mean serum concentrations of progesterone were determined by the contrast procedure of SAS. Differences between mean nonactivated or activated adenylyl cyclase activities, peak OT concentrations, tissue concentrations of OT, and ratio of luteal c-*jun*/18S RNA for saline or GnRH-treated heifers were tested for significance by *t*-test using Statgraphics (STSC, Rockville, MD).

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