Relaxin, Oxytocin, and Prostaglandin Effects on Progesterone Secretion from Bovine Luteal Cells during Different Stages of Gestation[1] (43144)

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Abstract. **To determine the effects of relaxin, oxytocin, and prostaglandin $F_{2\alpha}$ on progesterone secretion, bovine luteal cells from different stages of gestation were dispersed in Medium 199 with 200 units/ml penicillin, 1.0% kanamycin, 0.5% bovine serum albumin, and 400 units/ml collagenase. Cells (10^5) were cultured in 400 μl of Dulbecco's modified Eagle's medium and Ham's F-12 medium containing fetal bovine serum and antibiotics, in Falcon multiwell plates, in a humidified environment of 95% O_2 and 5% CO_2 at 37°C. Cells were cultured for 24 hr without treatment and thereafter with medium-hormone replacement every 24 hr. Progesterone was quantified from unextracted media by radioimmunoassay. Basal progesterone secretion after 24 hr was 1.81 ± 0.14, 1.76 ± 0.17, 0.54 ± 0.49, and 0.57 ± 0.21 pg/ml per viable luteal cell from 145-, 165-, 185-, and 240-day-old corpora lutea, respectively. Basal progesterone secretion increased ($P < 0.05$) with time in culture. Relaxin induced a dose-dependent (>100 ng/ml) increase in progesterone release, compared with the controls. Oxytocin and prostaglandin $F_{2\alpha}$ induced greater release ($P < 0.05$) of progesterone than relaxin at all stages of gestation, but progesterone release was dependent on the stage of gestation and the duration in culture. Luteinizing hormone (100 ng/ml) stimulated whereas 17β-estradiol (50 ng/ml) inhibited progesterone secretion by luteal cells at all stages of gestation examined. Relaxin obliterated the prostaglandin- and oxytocin-induced progesterone secretion by bovine luteal cells from 145 to 214 days of gestation. Thus, relaxin, cloprostenol, and oxytocin regulate progesterone production by cultured bovine luteal cells, but hormone secretion was dependent on the stage of gestation.** [P.S.E.B.M. 1990, Vol 195]

The bovine corpus luteum is the major source of progesterone for the maintenance of pregnancy; its removal in late gestation can result in abortion within 36 hr (1). The corpus luteum also produces oxytocin (2–5), prostaglandins (6), and relaxin (7). Exogenous oxytocin is luteolytic in cattle (8, 9), and it can stimulate the release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) after a single im injection of a high dose (10) or iv application of a moderate dose (11). The $PGF_{2\alpha}$ response to iv injected oxytocin is suppressed in pregnant heifers (10), whereas continuous infusion of oxytocin does not affect the pulsatile release of $PGF_{2\alpha}$ at the time of spontaneous luteolysis in cyclic animals (12). The luteolytic effects of oxytocin require a functional uterus (8, 9). Prostaglandins have a dual role in bovine luteal integrity, being both luteotropic (13, 14) and luteolytic (15–17). Cloprostenol, a potent analog of $PGF_{2\alpha}$, is luteolytic in the cow (17, 18). $PGF_{2\alpha}$ and analogs of it can stimulate abrupt oxytocin release during the estrous cycle (5). *In vivo* studies (19–23) and *in vitro* studies (24, 25) were interpreted to indicate that relaxin causes a significant change in progesterone, estrogen, and oxytocin secretion in late pregnant beef heifers. However, progester-

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one secretion from different stages of the estrous cycle seems unrelated to, or not dependent on, the release of prostaglandin (6).

This study was designed to determine the effects of relaxin, oxytocin (syntocinon), and a $PGF_{2\alpha}$ analog (cloprostenol) on progesterone release from cultured bovine luteal cells obtained from ovaries at different stages of gestation.

Materials and Methods

Hormones and Reagents. Purified porcine relaxin (3000 units/mg) was obtained from the ovaries of pregnant pigs and purified according to procedures described previously (26). A potent analog of $PGF_{2\alpha}$, cloprostenol sodium (Estrumate, 250 mg/ml), was obtained from Haver Lockhart ICI (Cheshire, Great Britian); 17β-estradiol, lyophilized, irradiated, and cell culture tested (lot 64F-8925), was obtained from Sigma Chemical Co. (St. Louis, MO). Highly purified bovine lutropin (LH) (APF 6000) was obtained from USDA Reproduction Laboratory (Beltsville, MD). Medium 199 (no. 2520), collagenase type 1-S (1050 units/mg; lot 26F-6830), fetal bovine serum Hybrimax (lot N2 125F4 6J09), kanamycin (10 mg/ml; lot 123F6828), tissue culture water (lot 036F4625), and Dulbecco's modified Eagle's medium/Ham's F-12 medium (lot 095F46081) were all obtained from Sigma Chemical Co.

Luteal Cell Dispersion and Culture. Corpora lutea were enucleated from the ovaries of pregnant cows at a local abattoir ($\cong$10 min after slaughter) and transported to the laboratory in ice-cold holding medium. The crown-rump length of the fetus associated with the corpus luteum was measured for determination of the age of the corpus luteum (27). Bovine luteal cells were dissociated (~90 min after slaughter) by using procedures described earlier (24). Viable cells (10^5) were cultured in 400 μl of culture medium in Falcon multiwell plates. Cells were cultured for 24 hr without hormone treatment, and this first 24 hr was designated day 0.

Culture medium from the first 24 hr without hormone treatment was assayed to establish basal levels of progesterone secretion. Treatments consisted of culturing cells with purified porcine relaxin (0, 10, 50, 100, 500, and 1000 ng/ml), syntocinon (20 mIU/ml), cloprostenol (1000 ng/ml), bovine LH (100 ng/ml), 17β-estradiol (50 ng/ml), or relaxin (1000 ng/ml) combined with syntocinon or cloprostenol. Cell viability was monitored daily by trypan blue exclusion of cells trypsinized from control wells. Medium was changed every 24 hr.

Radioimmunoassay of Progesterone in Culture Media. Progesterone in cell culture medium was quantified with minor modification of the procedures described (20, 28). The unextracted medium was diluted with phosphate-buffered saline, pH 7.2, and assayed. Samples were assayed in duplicate, using 200 μl of diluted medium. The sensitivity and inter- and intraassay coefficients of variation of the progesterone assay were 0.25 ng/ml, 6.8%, and 3.6%, respectively.

Statistical Analysis. In this study there were two replications of each stage of gestation. Each experimental treatment was replicated in three wells. Replicates (wells) were considered as experimental units. To obtain the effects of time, data were pooled for treatment across stage of gestation and, to obtain data on stage of gestation, data were pooled across all times for treatments. Data were analyzed by age, treatment, and time effects by using, where appropriate, analysis of variance, general linear model analysis, and Student's t test (29, 30).

Results

Corpus luteum weight ranged from 4.1 to 6.8 g. Luteal cells were classified as small (<10 μm in diameter), medium (10–25 μm in diameter), large (25–50 μm in diameter), or ultralarge (>70 μm in diameter). The proportions of large and ultralarge (>25-μm) cells, cell viability, and cell numbers were 19%, 98%, and 10 $\times$ 10^6 cells/g for 100-day-old corpora lutea and 6%, 90%, and 1.23 $\times$ 10^6 cells/g for 240-day-old corpora lutea, respectively. Cell viability after cell dissociation for all ages of corpora lutea exceeded 90% and was greater for cells obtained from the younger corpora lutea. In this study, a mixed population of luteal cells from corpora lutea of different gestational ages was used. After 48 hr in culture, cell viability of corpora lutea from early pregnancy was 90%, whereas cells obtained from corpora lutea of late gestation were less than 85% viable; at 72 hr, the viabilities were 86% and 75%, respectively.

Cultures of a mixed population of luteal cells released progesterone into the culture medium at levels inversely related to the age of the corpora lutea at the beginning culture (0 hr; Fig. 1 and Table I). After prolonged culture, however, cells obtained from late stages of gestation were able to release more progesterone than those obtained from early and mid-gestation (Fig. 1 and Table I). Basal progesterone secretion was least by the older luteal cells, but these cells released proportionally greater amounts of progesterone when co-cultured with relaxin, $PGF_{2\alpha}$ analog, and oxytocin than luteal cells from earlier stages of gestation (Table I). Luteal cells of ovaries from 185 days of gestation released twice as much progesterone as cells of ovaries from 145 and 165 days of gestation during 48-hr culture (Fig. 1).

Porcine relaxin added to the culture medium induced a dose-dependent increase of progesterone release into the culture medium after 48 hr (Fig. 2). Doses of relaxin greater than 100 ng/ml increased progesterone content in the spent medium, but lower doses had

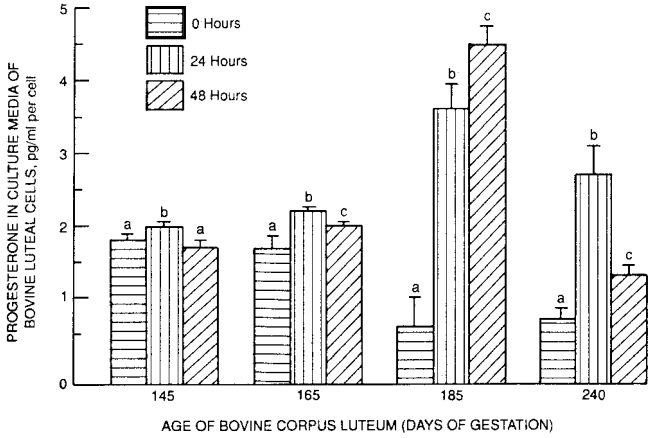

Figure 1. Progesterone (pg/ml/cell) release into culture media from bovine luteal cells from different stages of gestation. Cells (10^5 in 400 μl of culture media) were cultured with medium replacement every 24 hr. The first 24 hr of culture was designated 0 hr, the second replacement (48 hr in culture) was designated 24 hr, and the third replacement (72 hr in culture) was designated 48 hr. The experiment was repeated twice with three replicates per treatment. Values are mean ± SE. Bars with different letters differ ($P < 0.05$) within group.

Table I. Effect of Relaxin (RLX), Oxytocin (OT), and Cloprostenol ($PGF_{2\alpha}$ analog) on Progesterone Secretion by Cultured Bovine Luteal Cells from Different Stages of Gestation

Hormone	Progesterone in culture media (pg/ml per luteal cell)[a]		
	Early pregnancy (<145 days)	Middle pregnancy (165–185 days)	Late pregnancy (>240 days)
Control	1.84	1.46	1.07
RLX (1000 ng/ml)	1.94	2.40[b]	1.31
OT (20 mIU/ml)	2.22[b]	3.44[b]	1.95[b]
$PGF_{2\alpha}$ analog (1000 ng/ml)	2.23[b]	3.22[b]	1.86[b]
RLX + OT	2.17[b]	2.97[b]	1.64[b]
RLX + $PGF_{2\alpha}$ analog	2.17[b]	2.35[b]	1.79[b]
OT + $PGF_{2\alpha}$ analog	2.32[b]	3.20[b]	2.47[b]

[a] Average progesterone release by 10^5 luteal cells at 24, 48, and 72 hr of incubation. The number of replicates was three wells per treatment in duplicate. Pooled SE between treatment and pregnancy groups = 0.12 pg/ml per cell.
[b] $P < 0.05$ versus control within column.

no significant effect on progesterone secretion (Fig. 2). The amount of progesterone released into the medium increased with duration in culture; thus, relaxin (1000 ng/ml) increased progesterone content in the medium by 75% in the 48-hr cell cultures (Fig. 2).

Syntocinon, cloprostenol, and LH were more potent stimulators of progesterone release than relaxin (Fig. 3). After prolonged culture, luteal cells were more responsive to oxytocin than to LH and cloprostenol; syntocinon produced twice as much progesterone as did cloprostenol and 40% more than LH in 1.23×10^6

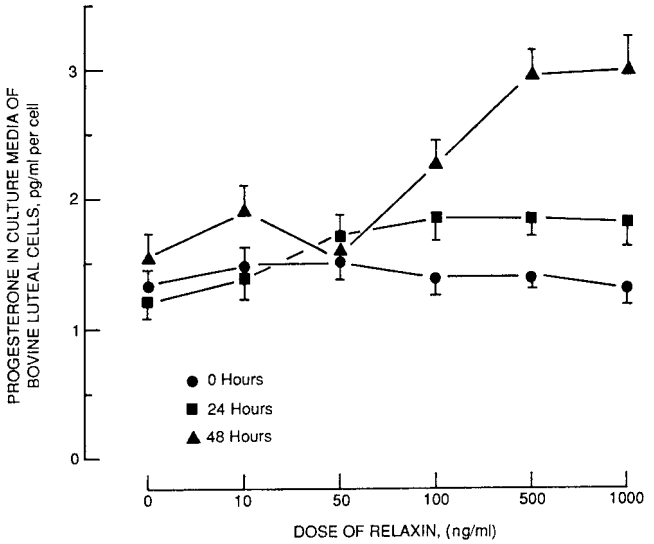

Figure 2. Effects of different doses of purified porcine relaxin (3000 units/mg) on progesterone (pg/ml/cell) release by the cultured bovine luteal cells at 0, 24, and 48 hr, averaged from incubations of four different gestational ages (Days 145, 165, 185, and 240) of corpora lutea. At 0 hr no relaxin was included in the culture medium. The experiment was repeated twice with three replicates per treatment for each gestational age. Data were pooled for time points per treatment. Values are mean ± SE.

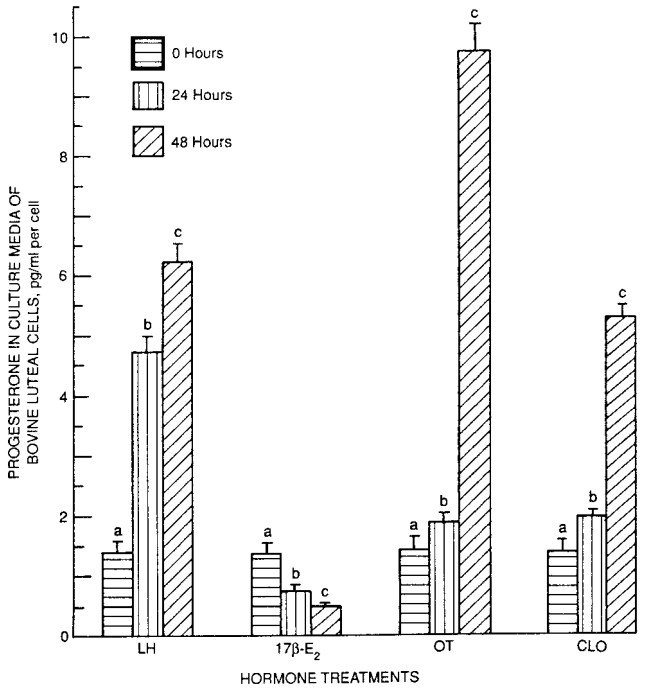

Figure 3. Effects of LH (100 ng/ml), 17β-estradiol (50 ng/ml) (17β-E_2); oxytocin (20 mIU/ml) (OT), and cloprostenol (1000 ng/ml) (CLO) on progesterone (pg/ml per bovine luteal cell) release into cell culture media at 0, 24, and 48 hr, averaged from incubations of four different gestational ages (Days 145, 165, 185, and 240) of corpora lutea. The experiment was repeated twice with three replicates per treatment for each gestational age. Data were pooled from time points per treatment. Values are mean ± SE. Bars with different letters differ ($P < 0.05$) within group.

cells for 48-hr culture medium (Fig. 3). LH stimulated the release of progesterone into cell culture media by as much as 57% above basal secretion after 24 hr in culture (Fig. 3). In these experiments, 17β-estradiol decreased progesterone secretion in all luteal cells from all stages of gestation, and the level of decreased progesterone secretion was dependent upon the duration of culture (Fig. 3). Combined cultures of relaxin with syntocinon or cloprostenol impaired syntocinon- and cloprostenol-induced release of progesterone into the culture medium (Fig. 4). A combination of cloprostenol and syntocinon, similarly, did not increase progesterone release above that seen for syntocinon alone (Fig. 4).

Discussion

The results from this study provide new information about the role of relaxin in the regulation of progesterone secretion by monodispersed bovine luteal cells. This study indicated that relaxin, oxytocin (syntocinon), and a potent $PGF_{2\alpha}$ analog (cloprostenol) induce specific increases in progesterone secretion from cultured bovine luteal cells. The steroidogenic response of these luteal cells to relaxin, syntocinon, and cloprostenol is dependent upon the stage of gestation at which the corpus luteum was harvested for cell dispersion, as well as the duration in culture. Unstimulated release of progesterone into the cell culture medium throughout the duration of culture diminishes as gestation advances. However, these cells respond differently to these three exogenous hormones by being able to secrete more progesterone into the medium during prolonged culture. Furthermore, the luteal cells from mid- and late gestation produced more progesterone during the entire 72 hr of culture, even though progesterone production was less during the first 24 hr.

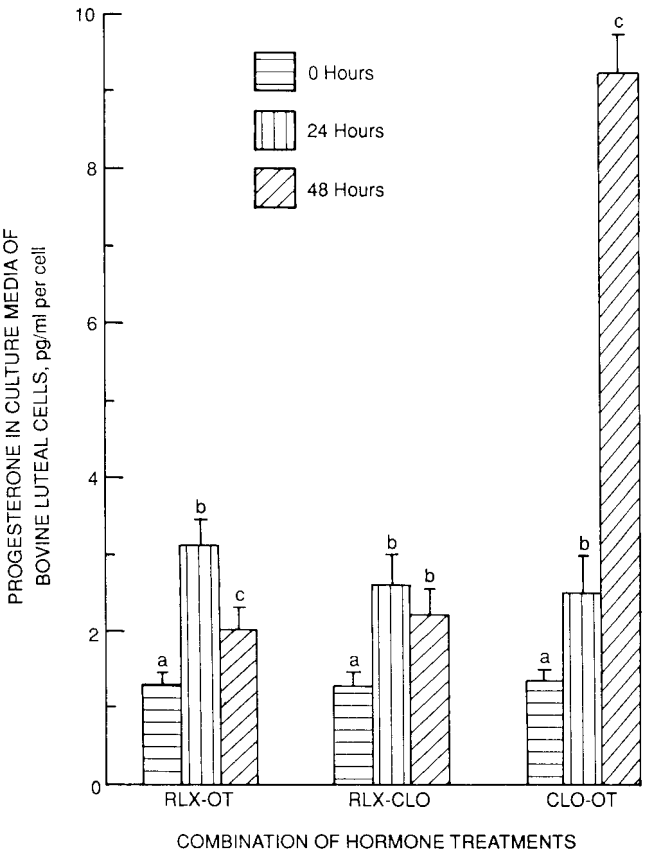

Figure 4. Progesterone (pg/ml per bovine luteal cell) release in response to relaxin (1000 ng/ml) (RLX) combined with oxytocin (20 mIU/ml) (OT) or with cloprostenol (1000 ng/ml) (CLO) and to oxytocin (20 mIU/ml) combined with cloprostenol (1000 ng/ml) at 0, 24, and 48 hr, averaged from incubations of four different gestational ages (Days 145, 165, 185, and 240) of corpora lutea. The experiment was repeated twice with three replicates per treatment for each gestational age. Data were pooled from time points per treatment. Values are mean ± SE. Bars with different letters differ ($P < 0.05$) within group.

In vivo studies in cattle indicate that during late pregnancy relaxin has a biphasic effect on progesterone secretion (18–23). Intravenous infusion of relaxin in low dosages (i.e., <500 μg/hr) is associated with greater peripheral blood concentrations of progesterone, whereas high infusion dosages (i.e., >600 μg/hr) or im injections of 1 or 2 mg of relaxin result in a significant acute decrease in progesterone secretion (20–22). The results from cultured bovine luteal cells presented here also show a biphasic effect of relaxin on progesterone secretion. In cell culture, a minimum threshold of relaxin (i.e., >100 ng/ml) induces increased progesterone release into the culture medium, whereas levels of relaxin less than 100 ng/ml had no effect on progesterone production. There is a major difference, however, in the *in vivo* and *in vitro* effects of relaxin on progesterone secretion in this species. In this *in vitro* model, higher dosages of relaxin (i.e., 100–1000 ng/ml) increased progesterone secretion, which was time and dose dependent, whereas *in vivo* high dosages of relaxin depress progesterone secretion.

It is currently hypothesized that progesterone synthesis in either the granulosa- or theca-derived large luteal cells is controlled in part by the Ca^{2+}-polyphosphoinositol C-kinase system, whereas synthesis in the numerous small theca-derived bovine luteal cells is controlled by LH and the cAMP system, which is "turned off" by high concentrations of Ca^{2+} (31–33). Thus, protein kinase C may selectively activate cytochrome P_{450}. Based on this hypothesis, the *in vivo* system might reflect the response of two types of luteal cells. The large bovine luteal cells, which are controlled primarily by Ca^{2+}-polyphosphoinositol C-kinase, may be activated only *in vivo*, whereas the small luteal cells, which are cAMP-dependent, will be activated both *in vivo* and *in vitro* in response to cAMP. Whether progesterone secretion, which is decreased by relaxin *in vivo* and increased by relaxin *in vitro*, is mediated indirectly through a Ca^{2+}-polyphosphoinositol C-kinase or directly by cAMP-driven progesterone release was not determined in the present study. This study confirms other reports that oxytocin and prostaglandins,

which *in vivo* decrease progesterone release in cattle (3, 17), are associated with increased progesterone release *in vitro* by dispersed luteal cells (34). In an earlier report (21), we hypothesized that relaxin, oxytocin, and prostaglandin have a feedback interaction in regulating bovine luteal integrity and progesterone secretion. The present study confirms that each of these hormones *in vitro* can affect progesterone secretion by bovine luteal cells from different stages of gestation. In *in vitro* as well as *in vivo* systems, relaxin alters oxytocin secretion (19, 24) and prostaglandin secretion (35) in cattle.

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