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Effects of Luteinizing Hormone and Prostaglandin $F_{2\alpha}$ on Gap Junctional Intercellular Communication of Ovine Luteal Cells Throughout the Estrous Cycle

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Cellular interactions mediated by contact-dependent pathways may be important to maintain luteal function. The objective of the present experiment was to evaluate the role of LH and prostaglandin $F_{2\alpha}$ (PGF) in regulation of contact-dependent, gap junctional intercellular communication (GJIC) of ovine luteal cells from several stages of luteal development. Corpora lutea (CL) obtained from superovulated ewes on days 5 (n = 7), 10 (n = 8), and 15 (n = 9) after estrus were dispersed with collagenase and cell types were separated by elutriation. Cells were plated as a mixed population (nonelutriated), or as small or large luteal cell fractions, and incubated in serum-free media containing no hormone, LH (100 ng/mL), PGF (100 ng/mL), LH + PGF, or dibutyryl cAMP (dbcAMP; 2 m*M*) for 18–24 h. Media were collected for evaluation of progesterone (P4) concentrations and replaced with media containing fluorescent dye. Then the rate of GJIC was evaluated by using the fluorescence recovery after photobleaching technique and laser cytometry. The rate of GJIC was determined for selected cells: small luteal cells in contact only with small luteal (S–S) cells; large luteal cells in contact only with small luteal (L–S) cells; and large luteal cells in contact only with large luteal (L–L) cells. LH increased ($p < 0.01$) GJIC for S–S on d 5 and 10 and for L–S cells across the estrous cycle, but did not affect GJIC for L–L cells. PGF increased ($p < 0.05$) GJIC for L–L cells on d 10 and 15, and decreased ($p < 0.05$) GJIC for S–S cells from d 5 and 10 of the estrous cycle. LH + PGF increased ($p < 0.05$) GJIC for S–S cells on d 5 and 10, and for L–S and L–L cells on d 10 and 15 of the estrous cycle. In addition, PGF diminished ($p < 0.05$) LH-stimulatory effects on GJIC for S–S cells from d 5 and 10, and for L–S cells from d 5 of the estrous cycle. Dibutyryl cAMP stimulated ($p < 0.05$) GJIC between all evaluated cell types across the estrous cycle. LH and dbcAMP stimulated ($p < 0.05$) P4 secretion by mixed and small luteal cell fractions, PGF alone did not affect basal P4 secretion, but LH + PGF stimulated ($p < 0.05$) P4 production by small luteal cells across the estrous cycle. PGF diminished ($p < 0.05$) LH-stimulatory effects on P4 production in mixed populations of luteal cells across the estrous cycle.

These data demonstrate that both luteal cell types communicate with each other, and the rate of communication was affected by LH, PGF, and dbcAMP. Modulation of gap junctional contact-dependent intercellular communication may be an important mechanism by which regulatory signals are transduced during luteal growth, differentiation, and regression in sheep.

Key Words: Cellular interactions; gap junctions; LH; $PGF_{2\alpha}$; corpora lutea; ewe.

Introduction

The corpus luteum (CL) is a transient endocrine gland that exhibits regular periods of growth, differentiation, and regression during each estrous cycle and pregnancy, and plays a major role in the reproductive process (Jablonka-Shariff et al., 1993; Grazul-Bilska et al., 1994; Reynolds et al., 1994; Zheng et al., 1994; Redmer and Reynolds, 1996).

The function of the CL depends on a balance between luteotropic and luteolytic factors (Niswender and Nett, 1994). In ewes, the major luteotropic agent is luteinizing hormone (LH), whereas the primary luteolytic factor is prostaglandin $F_{2\alpha}$ (PGF; Niswender and Nett, 1994). Multiple mechanisms appear to be involved in the regulation of luteal function. Numerous studies have evaluated the effects of LH and PGF on ovine luteal morphology, progesterone (P4) production, or intracellular signaling (Hansel et al., 1991; Niswender and Nett, 1994; Wiltbank, 1994). But little is known about the role they may play in the regulation of cellular interactions within luteal tissues.

The CL is a heterogeneous organ consisting of several cell types, which differ in morphological and physiological characteristics (Niswender and Nett, 1994). It has been suggested that luteal cells interact to maintain luteal func-

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tion (Schwall et al., 1986; Hansel et al., 1991; Redmer et al., 1991; Ackland, 1992; Grazul-Bilska, 1994, 1996b; Niswender and Nett, 1994; Del Vecchio et al., 1995). Cellular interactions may be mediated by several mechanisms, including humoral (endocrine or exocrine) and gap junctional pathways (Trosko et al., 1990). Gap junction-mediated intercellular communication is considered one of the most important mechanisms for coordination of cellular metabolism and function during growth and differentiation of organs and tissues (Loewenstein, 1991; Trosko et al., 1990; Holder et al., 1993). In a variety of tissues, function and structure of gap junctions are regulated by numerous factors, including hormones, growth factors and intracellular regulators (Saez et al., 1990; Stagg and Fletcher, 1990; Trosko et al., 1990; Redmer et al., 1991; Grazul-Bilska et al., 1994, 1996d). Structural and functional gap junctions have been identified in luteal tissues of several species (Redmer et al., 1991; Grazul-Bilska et al., 1994, 1996b,d; Mayerhofer and Garfield, 1995; Khan-Dawood et al., 1996). For bovine and ovine luteal cells, we have shown that contact-dependent gap junctional communication is affected by several regulators of luteal function (Redmer et al., 1991; Grazul-Bilska et al., 1994, 1996c,d).

The aim of this study was to evaluate the effects of luteotropic and luteolytic hormones on gap junctional intercellular communication (GJIC) of ovine luteal cell types from several stages of luteal development.

Results

Average number of CL/superovulated ewe was 15.1 ± 1.4 across the estrous cycle. Average CL weights were 253.3 ± 7.3 mg, 428.2 ± 12.8 mg, and 250.7 ± 6.9 mg on d 5, 10, and 15, respectively, and were greater ($p < 0.05$) on d 10 of the estrous cycle than on d 5 and 15, which were similar.

Ovine luteal cell types communicated with each other. LH increased ($p < 0.05$) the rate of GJIC for S–S cells from d 5 and 10, and for L–S cells across the estrous cycle (Table 1). PGF increased ($p < 0.05$) the rate of GJIC for L–L cells from d 10 and 15, but decreased ($p < 0.05$) the rate of GJIC for S–S cells from d 5 and 10 of the estrous cycle (Table 1). LH + PGF increased the rate of GJIC for S–S cells from d 5 and 10, and for L–S and L–L cells from d 10 and 15 of the estrous cycle (Table 1). However, the rate of GJIC was less ($p < 0.05$) in LH + PGF-treated cultures than LH-treated cultures for S–S cells from d 5 and 10, and for L–S cells from d 5 (Table 1). Dibutyryl cAMP ($p < 0.05$) stimulated GJIC for all evaluated cell types across the estrous cycle.

Across all cultures, no treatment effects on the rate of GJIC for cells which were not photobleached (positive control) or for cells which were photobleached but not in contact with other cells (negative control) were observed. The rate of dye transfer across treatments for these cells was negligible (0.03 ± 0.02% [$n = 4708$] for positive control and -0.05 ± 0 03 [$n = 229$] for negative control over the first 4 min of the assay).

Table 2 presents P4 concentrations in media from control cultures (no treatment) of mixed, small and large luteal cell fractions from d 5, 10, and 15 of the estrous cycle. Overall, P4 production was greater ($p < 0.05$) on d 10 than on d 5, which was greater ($p < 0.05$) than on d 15 of the estrous cycle.

Progesterone secretion by mixed and small luteal cell but not by large luteal cell fractions was affected ($p < 0.05$) by hormone treatments (Fig. 1). LH and dbcAMP increased ($p < 0.05$) P4 secretion by mixed and small luteal cell fractions across the estrous cycle (Fig. 1). PGF did not affect basal P4 secretion but decreased ($p < 0.05$) LH-stimulated P4 production by the mixed population of luteal cells across the estrous cycle (Fig. 1). In contrast, LH+PGF increased ($p < 0.05$) P4 secretion by the small luteal cell fraction across the estrous cycle (Fig. 1).

Discussion

The present data demonstrated that throughout the estrous cycle ovine luteal cells communicated with each other via contact-dependent mechanisms. Protein hormones (e.g., LH, hCG, FSH, TSH) and cAMP have been shown to affect gap junction function in their target organs and a variety of cell types (Larsen, 1989; Saez et al., 1990; Munari-Silem et al., 1991; Cronier et al., 1994; Pluciennik et al., 1994; Burghardt et al., 1995). CL appear to be similar to other organs because LH, PGF, and dbcAMP affected GJIC between luteal cells across the estrous cycle (Redmer et al., 1991; Grazul-Bilska et al., 1994, 1996d; present data). In the present study, the hormone treatments affected GJIC for all luteal cell types except for large luteal cells from the early luteal phase and for small luteal cells from the late luteal phase of the estrous cycle. The lack of responsiveness may be owing to incomplete differentiation of large luteal cells early in the cycle (Wiltbank et al., 1995), and the advanced regression of small luteal cells in the late luteal phase. In the late luteal phase, ovine small luteal cells seemed to be more fragile and more sensitive to enzyme digestion than large luteal cells. In the present study, small luteal cells comprised only about 60% of the steroidogenic cells in the late luteal phase, whereas in the earlier stages small luteal cells comprised about 88% of the steroidogenic cells. Similarly, a greater loss of small luteal cells during the dissociation process has been observed for CL from the late compared with the mid-luteal phase of the estrous cycle (Schwall et al., 1986; Braden et al., 1988). In addition, preferential loss of small luteal cells during luteolysis in ewes and cows has been reported (Archbald et al., 1981; O'Shea and Wright, 1985; Farin et al., 1986; Braden et al., 1988). This may suggest that during the luteolytic process first small and then large luteal cells decrease in number.

In the present experiment, LH enhanced GJIC between small luteal cells and between large and small luteal cells, but dbcAMP increased GJIC for all cell types. Stimulatory effects of LH and cAMP on GJIC of bovine luteal cell types

Table 1
Effects of LH, PGF, and dbcAMP on GJIC of Ovine Luteal Cell Types from Days 5 (n = 7 ewes), 10 (n = 8), and 15 (n = 9) of the Estrous Cycle[a]

Day of the estrous cycle	Cell types[b]	Treatments[c]				
		No treatment	LH	PGF	LH ± PGF	dbcAMP
5	S–S	1.6 ± 0.1(527)	2.8 ± 0.1(491)[d]	1.3 ± 0.1(486)[e]	2.2 ± 0.1(474)[d,f]	2.8 ± 0.1(467)[e]
5	L–S	0.8 ± 0.1(110)	1.5 ± 0.1(99)[d]	0.6 ± 0.1(90)	0.9 ± 0.1(97)[f]	1.1 ± 0.1(95)[e]
5	L–L	0.6 ± 0.1(60)	0.9 ± 0.2(69)	1.1 ± 0.2(57)	0.5 ± 0.1(63)	1.2 ± 0.1(66)[e]
10	S–S	2.0 ± 0.1(344)	3.3 ± 0.1(355)[d]	1.6 ± 0.1(327)[e]	2.6 ± 0.1(311)[d,f]	3.3 ± 0.1(340)[d]
10	L–S	1.2 ± 0.1(78)	1.8 ± 0.1(81)[d]	1.1 ± 0.1(77)	1.5 ± 0.1(70)[e]	1.8 ± 0.1(99)[d]
10	L–L	0.7 ± 0.1(80)	0.6 ± 0.1(68)	1.1 ± 0.1(84)[e]	1.3 ± 0.1(69)[d]	1.3 ± 0.1(65)[d]
15	S–S	2.0 ± 0.1(185)	2.0 ± 0.1(205)	1.7 ± 0.1(178)	1.9 ± 0.1(185)	3.0 ± 0.1(210)[d]
15	L–S	1.5 ± 0.1(207)	1.8 ± 0.1(160)[e]	1.5 ± 0.1(188)	2.4 ± 0.1(190)[d]	2.1 ± 0.1(177)[d]
15	L–L	0.8 ± 0.1(283)	0.6 ± 0.1(233)	1.5 ± 0.1(267)[d]	1.5 ± 0.1(278)[d]	1.2 ± 0.1(226)[e]

[a]Data (means ± SEM) are presented as the rate of fluorescence recovery (percentage of the prebleach value per min) calculated from values obtained during the first 4 min after photobleaching. Numbers in parentheses indicate number of cells that were evaluated within each group.

[b]S–S, small luteal cells in contact only with small luteal cells; L–S, large luteal cells in contact only with small luteal cells; L–L, large luteal cells in contact only with large luteal cells.

[c]Cells were incubated with or without LH (100 ng/mL), PGF (100 ng/mL), LH + PGF (100 ng/mL each), or dbcAMP (2 mM) for 16–24 h before analysis of GJIC.

[d]$P < 0.01$.

[e]$P < 0.05$ differ from No treatment within a row.

[f]$P < 0.05$ for LH + PGF compared with respective LH-treatment.

Table 2
Concentrations of Progesterone (ng/10,000 cells) in Control (No Treatment) Cultures of Luteal Cells from Days 5 (n = 7 ewes), 10 (n = 8), and 15 (n = 9) of the Estrous Cycle[a]

	Day of the estrous cycle		
Fraction	5	10	15
Mixed population[b]	9.2 ± 4.5	16.0 ± 3.7	2.1 ± 0.6
Small luteal cells[b]	1.0 ± 0.3	1.9 ± 0.5	0.1 ± 0.02
Large luteal cells[b]	10.8 ± 2.6	23.6 ± 3.4	2.3 ± 0.5

[a]Luteal cells were incubated in serum-free medium for 16–24 h.
[b]Within a row, means (± SEM) differ ($p < 0.05$), with d 10 > d 5 and d 5 > d 15.

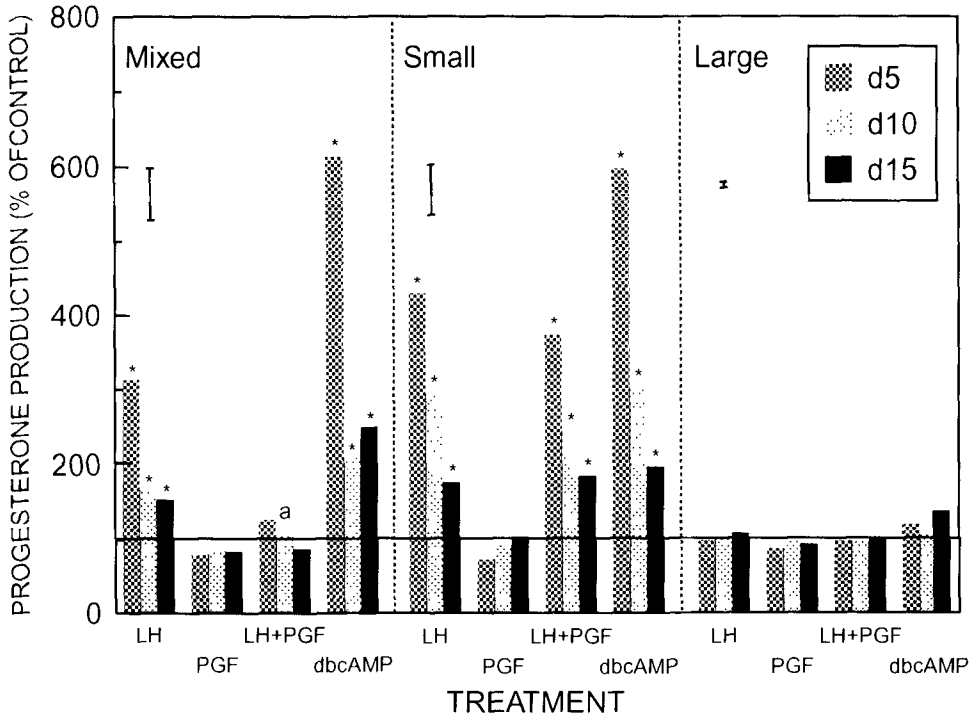

Fig. 1. Effects of LH, PGF, and dbcAMP on P4 production by mixed, small, and large luteal cells from d 5 (n = 7 ewes), 10 (n = 8), and 15 (n = 9) of the estrous cycle. Luteal cells were incubated with or without treatments for 16–24 h. Data are expressed as percent of control (100%, indicated by the solid horizontal line). For P4 production in control (no treatment) cultures, *see* Table 2. For Mixed, Small, and Large, the residual standard deviation was 68, 72, and 6% of control, respectively. *$p < 0.05$ compared with control (no treatment). [a] $p < 0.05$ for LH + PGF compared with respective LH treatment in mixed luteal cell cultures.

have been demonstrated (Redmer et al., 1991; Grazul-Bilska et al., 1996d). LH affects luteal function by binding to specific receptors present on plasma membranes of small and large luteal cells (Niswender and Nett, 1994; Wiltbank, 1994). After binding to its receptors, LH activates the cAMP-dependent protein kinase system, and increases intracellular cAMP and progesterone production in small luteal cells (Niswender and Nett, 1994). Although receptors for LH have been demonstrated for large luteal cells, no effects of LH on cAMP concentration or progesterone production of large luteal cells were observed (Hoyer and Niswender, 1985; Wiltbank, 1994). Although both small and large luteal cells contain a cAMP-dependent protein kinase system, and in both cell types intracellular concentrations of cAMP may be enhanced by adenylate cyclase activators, only small luteal cells exhibit an increase in progesterone production in response to elevated cAMP (Hoyer and Niswender, 1985). Therefore, it is very likely that LH enhances GJIC through activation of cAMP-dependent protein kinase (Grazul-Bilska et al., 1996d). Although in large luteal cells the cAMP-dependent protein kinase system is not involved in regulation of progesterone

production, it may be important to establish and maintain GJIC. But in small luteal cells the cAMP-dependent protein kinase system seems to be involved in both regulation of GJIC and progesterone secretion. These and other data demonstrate that, in ovine CL, the cAMP-dependent protein kinase system is involved in control of cellular interactions.

LH + PGF treatment increased GJIC between specific luteal cells across the estrous cycle. These effects may be due to LH or PGF stimulatory effects on GJIC between specific cell types, i.e., LH alone increased GJIC for S–S cells, and the same effect was observed for LH + PGF treatment, or PGF alone increased GJIC for L–L cells and the same effect was observed for LH + PGF. PGF alone increased or decreased GJIC between specific luteal cells and, in addition, decreased LH-stimulated GJIC between small and between large and small luteal cells. For bovine luteal cell types, PGF increased GJIC at the mid and/or late luteal phases of the estrous cycle (Redmer et al., 1991; Grazul-Bilska et al., 1996c). The differences for PGF effects on GJIC of specific luteal cells from cows or sheep may reflect different responsiveness of bovine versus ovine luteal cells to PGF in vitro, as reported before (Pate, 1994). These data suggest that PGF is involved in the regulation of gap junction-mediated cellular nteractions within luteal tissues. In some other organs such as myometrium, trachea, or bone, prostaglandins are also involved in the regulation of gap junction function (Garfield et al., 1980; Agrawal and Daniel, 1986; Shen et al., 1986).

PGF has been shown to be a major luteolytic factor in sheep (Niswender and Nett, 1994). PGF binds to its receptors present on luteal cell plasma membranes (Stormshak et al., 1987; Wiltbank, 1994; Custer et al., 1995). PGF may activate second messengers such as inositol phosphates, diacylglycerol, calcium, and the protein kinase C system (Stormshak et al., 1987; Wiltbank et al., 1991; Niswender and Nett, 1994). The mechanism of PGF action on GJIC has not been investigated, but it has been demonstrated that second messengers, which may be activated by PGF, are involved in the regulation of gap junction function in a variety of tissues including CL (Klaunig and Ruch, 1990; Saez et al., 1990; Stagg and Fletcher, 1990; Grazul-Bilska, 1994, 1996c).

Increase of GJIC between large luteal cells from the mid and late luteal phases of the estrous cycle in the presence of PGF may be associated with the luteolytic process, which may be initiated as early as in the mid luteal phase in sheep (Inskeep, 1973). It has been hypothesized that only large luteal cells may be a target for PGF, since they possess the majority of PGF receptors. The luteolytic signal may be transduced to small luteal cells through gap junctions or other mechanisms (Niswender and Nett, 1994; Pate, 1994). On the other hand, receptors for PGF have also been detected on small luteal cells, although the affinity of these receptors for PGF is lower than that of large luteal cell PGF receptors (Balapure et al., 1989; Chegini et al., 1991; Niswender and Nett, 1994). Therefore, it is possible that PGF may also act directly on small luteal cells. Inhibitory effects of PGF on GJIC between small luteal cells was observed in the present experiment. From these data, it is tempting to speculate that the PGF-stimulated increase in the rate of GJIC among large cells is important for transducing luteolytic signals across the CL, while at the same time PGF may inhibit communication among small luteal cells. Lack of PGF effects on GJIC between large luteal cells from the early luteal phase may also help explain why PGF is less efficacious in inducing luteolysis early in the cycle (Inskeep, 1973).

Progesterone secretion by luteal cell types was the greatest at the mid luteal phase, and less at the early and late luteal phases, which follows the pattern of P4 secretion in vivo (Goodman, 1994). The present data suggests interactions between luteal cell types in P4 production. The predicted progesterone secretion by mixed luteal cells based on the rate of P4 production by small and large luteal cell fractions is much lower than the actual rate of P4 secretion. For example, the mixed population of luteal cells from d 5 and 10 contains about 88% small luteal cells and 12% large luteal cells, which individually would produce about 2.2 ng/10,000 cells per 20 h on d 5 and 4.5 ng/10,000 cells per 20 h on d 10. The actual rate of progesterone production by mixed luteal cell cultures, however, was 9.2 ng/10,000 cells per 20 h on d 5 and 16 ng/10,000 cells per 20 h on d 10. Similarly, for cells from d 15, the predicted P4 secretion was about 1 ng/10,000 cells per 20 h, but the actual rate of progesterone production was 2.1 ng/10,000 cells per 20 h. These data agree with previous reports of interactions between ovine and bovine luteal cell types in P4 secretion (Harrison et al., 1987; Grazul-Bilska et al., 1991, 1994; Del Vecchio et al., 1995).

In the present experiment, the effects of LH, dbcAMP and PGF on P4 secretion by mixed, small, or large luteal cells were similar to those previously reported (Fitz et al., 1982; Fletcher and Niswender, 1982; Rodgers et al., 1983; Hoyer et al., 1984; Hoyer and Niswender, 1985; Harrison et al., 1987; Conley and Ford, 1989; Grazul-Bilska et al., 1991, 1995; Wiltbank et al., 1991; McCann and Flint, 1993; Niswender and Nett, 1994; Wiltbank, 1994). However, PGF inhibitory effects on P4 production by large luteal cells have been reported (Fitz et al., 1984; Wegner et al., 1990; Wiltbank et al., 1990). These discrepancies may be due to different culture conditions and/or different treatment of ewes. In addition, in the present and other studies PGF decreased LH-stimulated P4 secretion by mixed but not small or large luteal cells, and did not affect basal progesterone secretion (Evrard et al., 1978; Fletcher and Niswender, 1982; McCann and Flint, 1993). This suggests that both cell types must be present for PGF to exert its negative effects on P4 secretion. Several investigators have suggested that large–small luteal cell and/or steroidogenic–endothelial cell interactions are required for

PGF to influence luteal function (Fitz et al., 1982; Michael et al., 1994; Niswender and Nett, 1994; Pate, 1994; Girsh et al., 1995).

In this in vitro experiment, we did not observe a relationship between the rate of GJIC and basal P4 secretion. The basal rates of P4 secretion differed on d 5, 10, and 15 of the estrous cycle but rates of GJIC did not. In addition, expression of the gap junctional protein connexin 43 (Cx43) in cultured ovine luteal cells was similar across the estrous cycle (Grazul-Bilska, unpublished). In contrast, for cultured bovine luteal cells, the rate of GJIC and P4 secretion were greatest early in the cycle and decreased as the estrous cycle progressed (Grazul-Bilska et al., 1996b,d). However, in vivo, expression of Cx43 in ovine and bovine CL was greater early in the cycle than in later stages of luteal development (Grazul-Bilska et al., 1996a,c). In addition, in bovine CL the rate of GJIC, Cx43 expression in vivo and in vitro, P4 secretion by luteal cells, and luteal concentrations of P4 (Grazul-Bilska et al., 1996b), and in ovine CL, Cx43 expression in vivo, P4 secretion by luteal cells and luteal concentrations of P4 (Grazul-Bilska et al., 1996a; present data; Grazul-Bilska, unpublished) were lowest during the late luteal phase of the estrous cycle. This suggests a relationship between the luteolytic process and gap junction function. In addition, the potential relationship between ovarian steroids and luteal gap junctions seems worthwhile to investigate since estrogens and progesterone are involved in the regulation of gap junction function as well as connexin expression in ovarian follicles and uterine tissues (Burghardt and Anderson, 1981; Petrocelli and Lye, 1993; Risek et al., 1995). We would also like to note that other connexins, such as Cx26 and Cx32, are likely to be important indicators of gap junction function in sheep CL. We have recently immunolocalized Cx26 and Cx32 in ovine luteal tissues (Grazul-Bilska, unpublished).

In the present experiment, luteal cells from superovulated ewes were used. Although several studies demonstrated that the superovulated ewe provides a model that is qualitatively similar to the normal ewe, a few differences in luteal function between superovulated vs normal ewes have been reported (McClellan et al., 1975; Hild-Petito et al., 1987). Therefore, differences in responsiveness of ovine luteal cells vs bovine luteal cells may be due not only to species, but also to animal treatments. However, comparison of the rate of GJIC of luteal cells and gap junction protein expression in luteal tissues of superovulated vs normal ewes requires further study.

In conclusion, LH increased GJIC between small and large and between small luteal cells, whereas PGF increased GJIC between large luteal cells and decreased GJIC between small luteal cells. Therefore, intercellular communication among luteal cells is likely to be very important during luteal development, differentiation and regression.

Materials and Methods

Reagents

For cell cultures, Dulbecco s Modified Eagle's Medium (DMEM), Ham's F-10 medium, Ca^{2+} and Mg^{2+}-free Hank's balanced salt solution, fetal bovine serum, calf serum, crystalline bovine insulin, trypan blue stain (0.4%), and penicillin-streptomycin (10,000 U penicillin G sodium salt and 10,000 μg streptomycin sulfate/mL) were purchased from Gibco (Grand Island, NY). Bovine serum albumin (fraction V), dimethylsulfoxide (DMSO), transferrin, hydrocortisone, etiocholan-3β-ol-17-one, nitroblue tetrazolium, PGF, dbcAMP, and NAD^+ were purchased from Sigma (St.Louis, MO). 5-carboxy-fluorescein diacetate acetoxymethyl ester (CFDA-AM) was purchased from Molecular Probes (Eugene, OR). LH (NIADDK oLH-25) was a gift from the NIH Animal Hormone Program and the National Hormone and Pituitary Program (Beltsville, MD). For radioimmunoassay, P4 standard was purchased from Sigma, and tritiated P4 from DuPont/New England Nuclear Products (Boston, MA). The P4 antibody (GDN 337) was kindly supplied by G. D. Niswender (Colorado State University, Fort Collins, CO). For induction of superovulation, follicle stimulating hormone (FSH-P) was purchased from Schering (Kenilworth, NJ).

Dissociation of Luteal Cells

Ovaries were collected from superovulated ewes on d 5 ($n = 7$), 10 ($n = 8$), and 15 ($n = 9$) after estrus. Superovulation was induced as described (Grazul-Bilska et al., 1991; Jablonka-Shariff et al., 1994; protocols for this experiment and animal care were approved by the IACUC). Corpora lutea were dissected from the ovaries and dissociated by using collagenase as described previously (Grazul-Bilska et al., 1991). After dissociation, a portion of the luteal cells was separated into fractions enriched with small or large luteal cells by using elutriation (Grazul-Bilska et al., 1991). The mixed (nonelutriated) population of luteal cells contained 87.8 ± 1.3% small and 12.2 ± 1.3% large luteal cells (nonsteroidogenic cells are not included) on d 5 and 10, and 59.8 ± 6.3% small and 40.2 ± 6.3% large luteal cells on d 15 of the estrous cycle. The small luteal cell fraction was not contaminated by the presence of large luteal cells, but the large luteal cell fraction contained 18.3 ± 1.6% (7–27%) small luteal cells on d 5 and 10, and 3.5 ± 1.2% (1–9%) small luteal cells on d 15 of the estrous cycle. Across the estrous cycle, viability of luteal cells was 89.1 ± 0.9% before elutriation and was 76.2 ± 2.7% for small and 76.6 ± 4.1% for large luteal cell fractions after elutriation. Luteal cells were resuspended in plating medium (DMEM containing 1% [v/v] fetal bovine serum, 1% [v/v] calf serum, and antibiotics [100 U penicillin and 100 μg streptomycin/mL]) and preincubated for 24-48 h in 35-mm petri dishes at a concentration which resulted in subconfluent cultures after 24 h (3×10^5 mixed luteal cells/dish; 3.5×10^5 small luteal

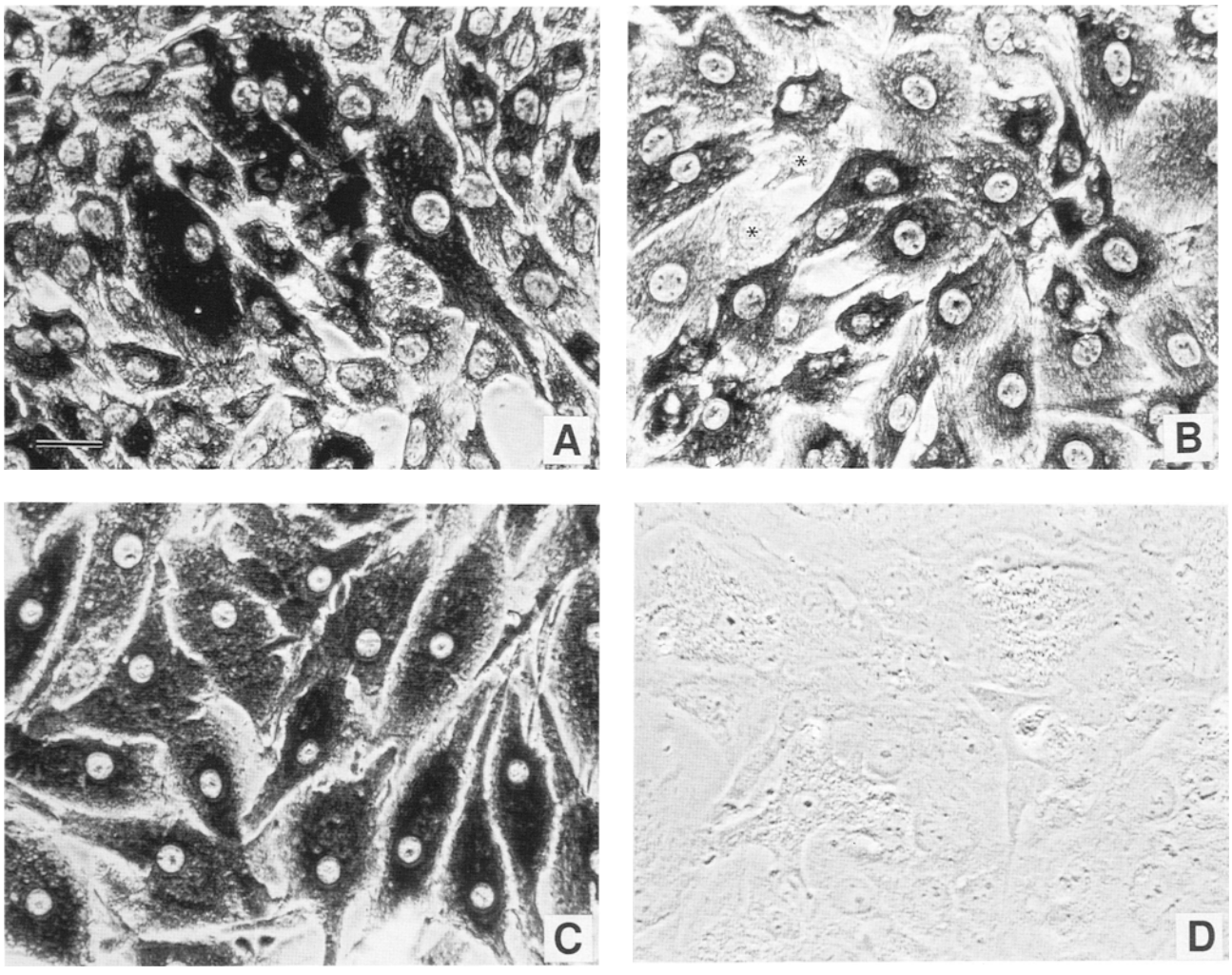

Fig. 2. Localization of 3β-HSD in mixed **(A)**, small **(B)**, and large **(C)** luteal cell cultures, and **(D)** control staining. Note localization of formazan granules (dark color) within the cytoplasm of steroidogenic small and large luteal cells, and absence of granules in nonsteroidogenic cells (asterisks). Staining was similar across the estrous cycle; micrographs are of cultured cells obtained from d 10 of the estrous cycle. Bar = 20 μm.

cells/dish; 2.5×10^5 large luteal cells/dish). All incubations were conducted at 37°C in a humidified atmosphere (5% CO_2 and 95% air). After preincubation, plating medium was changed to serum-free medium containing: no treatment (control), LH (100 ng/mL), PGF (100 ng/mL), LH + PGF (each 100 ng/mL), and dbcAMP (2 m*M*). The dose of each factor was established on the basis of preliminary and previous experiments (Grazul-Bilska et al., 1991). In preliminary experiments, mixed populations of luteal cells (n = 3 ewes) were plated as described above, and incubated in serum-free medium with 0, 1, 10, and 100 ng/mL LH or PGF or with 0, 0.5, 2, and 4 m*M* dbcAMP for 24 h. Medium was collected for P4 determination and cells evaluated for the rate of GJIC as described below. The most stimulatory dose on P4 production and/or GJIC was chosen for the current study. Dibutyryl cAMP, known as a factor that stimulates GJIC, was used in this experiment as a positive control (Grazul-Bilska et al., 1996d). For each cell type culture, three dishes were assigned to each treatment. After 16–24 h of incubation with treatments, media were collected for determination of P4 concentrations, and contact-dependent intercellular communication was measured by the fluorescence recovery after photobleaching (FRAP) technique, as described below.

Analysis of Contact-Dependent Intercellular Communication

GJIC among contacting luteal cells in culture was determined using the FRAP method (Wade et al., 1986; Redmer et al., 1991; Grazul-Bilska et al., 1996d). Briefly, after incubation with treatments, medium was removed from each dish, and fresh serum-free medium containing the fluorescent probe CFDA-AM (15 μ*M*) was added. After a 15-min incubation (22°C), dishes were rinsed three times with serum-free medium to remove excess CFDA-AM. Dishes were then placed onto the interactive laser cytometer (Meridian Instruments, Okemos, MI), and three fields (180 × 180 μm/field) on each dish were identified for scanning.

For each field, 4–12 cells were selected and analyzed for initial fluorescence intensities. Immediately after measurement of initial fluorescence, the fluorescent probe was photobleached in 2–8 selected cells in each field, as described by Redmer et al. (1991). Photobleaching caused a reduction of fluorescence to 30–60% of the initial fluorescence intensity value (100%). To determine the rate of FRAP, the fluorescence intensity of all selected cells was quantified every 2 min for 10 min after photobleaching. Data are presented as the rate of fluorescence recovery (percentage of the prebleach value per min) calculated from values obtained during the first 4 min after photobleaching. The total number of luteal cells evaluated per ewe was 579 ± 43.

Identification of Luteal Cell Types

Initial identification of steroidogenic luteal cells in culture was based on the characteristic granular appearance of the cytoplasm. Morphological classification was confirmed for two dishes of each fraction from each culture by staining for Δ5-3β-hydroxysteroid dehydrogenase (3β-HSD; a marker of steroidogenic cells), as described previously (Grazul-Bilska et al., 1996b). The majority of the cells in the culture dishes were steroidogenic (Fig. 2, *previous page*). For all cultures, the rate of GJIC was determined only for steroidogenic cells, as follows: small luteal cells in contact only with small luteal (S–S) cells, large luteal cells in contact only with small luteal (L–S) cells, and large luteal cells in contact only with large luteal (L–L) cells.

Progesterone RIA

Progesterone concentrations in unextracted luteal-conditioned media were measured by RIA, as previously reported (Grazul-Bilska et al., 1991; Redmer et al., 1991). The intra- and interassay coefficients of variation were 4.8 and 8.5%, respectively.

Statistical Procedures

Data for luteal weight, basal P4 secretion, and effects of treatments on the rates of GJIC and P4 secretion were analyzed by using the General Linear Models analysis of variance procedures with day of the estrous cycle (luteal weight and basal P4 secretion) or day of the estrous cycle, treatment within day and their interactions (split-plot, the rate of GJIC and P4 secretion) included in the model (SAS User's Guide, 1985; Grazul-Bilska et al., 1996d). When an F-test was significant ($p < 0.05$), differences between specific means were evaluated by Bonferroni's multiple comparison procedure (Kirk, 1982).

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