Hormonal regulation of connexin-43 in baboon corpora lutea

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Abstract

The synthesis and secretion of progesterone in the corpus luteum are regulated by both endocrine and paracrine/autocrine factors which affect the steroidogenic cells. Evidence suggests that these cells communicate via cell-cell junctional proteins, the connexins. Previously we have shown that connexin-43 is expressed in both human and baboon (*Papio hamadryus anubis*) corpora lutea, with differential expression throughout luteal development, but is not detectable in corpora albicantia. We have examined the effect of human chorionic gonadotropin (hCG), oxytocin, clomiphene citrate and the anti-progesterone onapristone on expression of connexin-43 protein in the early luteal phase 1–5 days after the mid-cycle luteinizing hormone (LH) surge (LH+1–5 days), the mid-luteal phase 6–10 days after the LH surge (LH+6–10 days), and the late luteal phase 11–15 days after the LH surge (LH+11–15 days) in corpora lutea obtained from normal adult cycling females. Connexin-43 was localized by immunohistochemistry in cultured cells from all the three stages. Western blot analysis of the treated cells indicated the presence of two bands at 43 and 45 kDa. The band at 45 kDa was found to be phosphorylated connexin-43, indicating the presence of functional gap junctions. hCG

(10 IU/ml) stimulated the expression of connexin-43 throughout luteal development; however, maximum expression occurred in the early luteal phase with a significantly greater expression of the non-phosphorylated protein. In contrast, in the mid-luteal phase, the expression of the phosphorylated protein was predominant. Oxytocin (200 mU/ml) also stimulated connexin-43 expression throughout luteal development with similar effects on the phosphorylated and non-phosphorylated protein in the early and mid-luteal phase; however, compared with hCG, oxytocin had a greater effect on mid-luteal phase connexin-43 expression. In the presence of both hCG and oxytocin, the expression of connexin-43 was significantly higher than the control only in the late luteal phase. Both clomiphene citrate and onapristone suppressed connexin-43 expression, and concomitant addition of hCG did not counteract their effect. In the context of our previous studies, it is concluded that, together with LH/hCG and the steroid hormones, oxytocin is involved in cell-cell contact-dependent communication in the corpus luteum.

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Introduction

Intimate cell–cell cooperation occurs between the steroidogenic cells of the corpus luteum for the synthesis of progesterone (Harrison *et al.* 1987, Redmer *et al.* 1991, Del Vecchio *et al.* 1994, 1995, Grazul-Bilska *et al.* 1994, 1996, Khan-Dawood *et al.* 1996a). We have proposed that contact-dependent communication involving intercellular junctions is of significant importance in these cells. We have identified gap junctions in the baboon and human corpora lutea by electron microscopy (Khan-Dawood *et al.* 1996b). Connexin-43, an integral component of the connexin that forms gap junctions (Beyer *et al.* 1990), was also immunohistologically localized in human and baboon corpora lutea (Khan-Dawood *et al.* 1996b). A significant observation of our studies was that the level of connexin-43 determined by Western blot analysis varied

throughout the various developmental stages of the corpus luteum, with peak levels in the mid-luteal phase, when this tissue is maximally active. None was detectable in the corpus albicans (Khan-Dawood *et al.* 1996b).

Luteinizing hormone (LH) plays an indispensable role in the activity of the corpus luteum (Yen 1986); however, several other factors produced by the cells of the corpus luteum may also be significantly involved in regulating progesterone synthesis (Dawood *et al.* 1992). Oxytocin (OT) is one such factor that is present in the steroidogenic cells of the human (Khan-Dawood 1987a) and baboon (Khan-Dawood 1987b) corpus luteum. OT receptors are present in luteal tissue (Khan-Dawood & Dawood 1993); OT is synthesized by luteal cells (Ivell *et al.* 1990) and is biologically active (Khan-Dawood *et al.* 1995). The levels of the peptide in the corpus luteum vary throughout the luteal phase, with maximum levels in the mid-luteal phase

(Dawood & Khan-Dawood 1986, Khan-Dawood *et al.* 1988). These observations collectively suggest that OT may be of importance in the development and demise of the corpus luteum.

To examine the role of OT in the corpus luteum further, in this study we have examined the effect of human chorionic gonadotropin (hCG), OT, clomiphene citrate (CC) and the progesterone antagonist onapristone (ZK) on the expression of the gap junctional protein connexin-43 by baboon corpus luteum cells in culture from the early (LH surge+1–5 days), mid- (LH surge+6–10 days) and late (LH surge+11–15 days) luteal phases.

Materials and Methods

Tissue collection

The study was approved by the Institutional Review Board for Animal Experimentation, University of Texas Health Science Center at Houston, and was carried out in accordance with the principles and procedures described in the *Guide for the Care and Use of Laboratory Animals* as approved by the National Institutes of Health (NIH 80–23). Corpora lutea were obtained at laparotomy by lute-ectomy from a group of adult female cycling baboons (*Papio hamadryus anubis*) with well-defined menstrual cycles and weighing between 14 and 17 kg as previously described (Khan-Dawood *et al.* 1988). The menstrual cycles were monitored daily by the appearance and score of the perineal tumescence and serum progesterone following detumescence. The day of perineal detumescence was equated to the day of the LH surge. Days 1–5 after the mid-cycle LH surge were classified as the early luteal phase, days 6–10 after the LH surge were the mid-luteal phase, and days 11–15 after the LH surge were the late luteal phase. A total of nine corpora lutea, three each from the early, mid- and late luteal phases, were obtained. The tissues were immediately processed for cell culture.

Materials

Connexin-43 antibody (Dr Robert Garfield, University of Texas Medical School, Galveston, TX, USA) is a polyclonal antibody raised in rabbits against the cytoplasmic C-terminal domain consisting of the amino acid sequence from 346 to 360 of the rat and human connexin-43 sequence. This antibody was also utilized in previous studies (Khan-Dawood *et al.* 1996b). The secondary antibody, biotinylated goat anti-rabbit IgG, was obtained from Transduction Laboratories (Lexington, KY, USA). Sucrose, 3*N*-morpholinopropanesulfonic acid, phenyl-methylsulfonyl fluoride (PMSF), dithiothreitol, CaCl₂, fetal bovine serum (FBS), clomiphene citrate, sodium orthovanadate, NaF and sodium pyrophosphate were

obtained from Sigma Chemical Co., St Louis, MO, USA. Trypsin containing 2 mM EDTA, trypsin inhibitor, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 (DMEM/F-12) and Matrigel were obtained from Life Technologies, Gaithersburg, NJ, USA. Insulin–transferrin–selenium–bovine serum albumin (ITS⁺) was obtained from Collaborative Biomedical Products, Bedford, MA, USA. Collagenase type IV (132 U/mg) was obtained from Worthington Biochemicals, Freehold, NJ, USA. Alkaline phosphatase MB grade (1000 U/ml) was obtained from Boehringer-Mannheim Biochemicals, Indianapolis, IN, USA. hCG and OT were gifts from Organon Inc., West Orange, NJ, USA and Sandoz, Basle, Switzerland respectively. Onapristone (ZK 98-299) was a gift from Schering AG, Berlin, Germany.

Luteal cell culture

The corpora lutea were dispersed in DMEM/F-12 containing 50 U/ml collagenase, 0.15 mg/ml DNase and 4.7 mg/ml HEPES for 2 h at 37 °C. After collagenase treatment, the supernatant with the dissociated cells was centrifuged at 200 *g* for 5 min. The cells were washed three times. The final pellet was suspended in DMEM/F-12 containing ITS⁺ and 1% heat-inactivated FBS. The remaining undissociated tissue was further dispersed mechanically with a Pasteur pipet and centrifuged. Cells in the supernatants were combined, and an aliquot was counted in a hemocytometer. The viability of the cells was determined using the trypan blue-exclusion test (Tennant 1964), and 92 ± 2% (*n*=9) of the cells were found to be viable.

Effect of hCG, OT, clomiphene citrate and onapristone on the expression of connexin-43

Luteal cells from each corpus luteum from each phase (early, mid- and late luteal phases) were cultured in triplicate for 4 days. Cells (100 000/well) were plated in 24-well culture dishes (Falcon, Oxnard, CA, USA) coated with the basement membrane, Matrigel (diluted 1:3, 40 µl/well) and cultured in 1 ml DMEM/F-12 medium containing ITS⁺ and 1% heat-inactivated FBS at 37 °C in 5% CO₂ and air. The cells attached within 24 h; the FBS-containing medium was then replaced with medium without FBS, and the cells were cultured for a further 24 h. Cells were treated with hCG, OT, clomiphene citrate or onapristone, or hCG and OT, hCG and clomiphene citrate, or hCG and onapristone for 2 days, with medium being replaced every 24 h. At the end of the 2 days the cells were trypsinized (0.01% trypsin and 2 mM EDTA), and an aliquot was removed for protein determination (Lowry *et al.* 1951). The remainder were lysed in buffer (O'Farrell 1975) and processed for Western blot analysis. In initial experiments,

the appropriate concentrations of hCG and OT to be used were determined on the basis of our previous studies (Khan-Dawood *et al.* 1988). Thus 10, 25 and 50 IU/ml hCG and 50, 100 and 200 mU/ml OT were evaluated using cells from the early luteal phase corpora lutea ($n=3$).

Western blot analysis of connexin-43

Cultured cells were lysed in buffer (O'Farrell 1975) after trypsin treatment. The buffer contained 1 mM $MgCl_2$, 0.01 U/ml aprotinin, 5 mM PMSF and 1 mM $CaCl_2$. After sonication for 20 s, samples were boiled for 5 min, and 10 μ g protein samples were electrophoresed on an SDS-12% polyacrylamide gel. After transfer to nitrocellulose membranes (Hybond-C Super, Amersham, Arlington, IL, USA), reaction with the primary antibody at 1 μ g/ml (a dilution of 1:1000) for 16 h at 4 °C and biotinylated secondary antibody (0.5 μ g/ml, 1:2000 dilution), the reaction products were detected using the Renaissance chemiluminescence procedure essentially as described by the manufacturer (Dupont-NEN, Boston, MA, USA). Prestained SDS-PAGE standards (Bio-Rad, Richmond, CA, USA) were run in parallel with each sample. To determine if connexin-43 was expressed in a phosphorylated form, 20 μ g protein from hCG- (10 IU/ml) and OT- (200 mU/ml) treated cells were incubated in the presence or absence of 20 U alkaline phosphatase for 4 h at 37 °C in the absence or presence of alkaline phosphatase inhibitors (1 mM sodium orthovanadate, 10 mM NaF and 10 mM sodium pyrophosphate). The samples were heated at 60 °C for 5 min to inactivate the enzyme, and aliquots equivalent to 10 μ g protein were analyzed by Western blot analysis as described (Musil & Goodenough 1993).

Immunohistological localization of connexin-43 in cultured cells

Luteal cells from each phase (early, mid and late) of the luteal cycle were cultured on Matrigel-coated four-chamber glass slides for 4 days. After a rinse with PBS, pH 7.2, the cells were fixed with 99.9% methanol and incubated for 16 h at 4 °C with the connexin-43 antibody at a dilution of 1:200. After several washes, the cells were treated with the secondary antibody, and the antigen was visualized using 3,3'-diaminobenzidine as the chromogen.

Analysis of data

The Western blots of three different corpora lutea from each phase were analyzed by determining band intensities at 43–45 kDa using a PhosphorImager Series 400 (Molecular Dynamics, Sunnyvale, CA, USA). Bands at 43 kDa and the phosphorylated band at 45 kDa (43P kDa)

were also analyzed separately. Image Quant 3.2 software was utilized. The densitometric values obtained for total connexin-43 were statistically analyzed using a one-way ANOVA to compare untreated and treated cells ($n=3$). The levels of connexin-43 and connexin-43P were also compared both with the control untreated cells and within each treatment group. Significant differences between samples were determined using Student–Newman–Keuls test. Differences were considered to be significant at $P \leq 0.05$.

Photomicrographs were obtained of the immunohistological slides at a magnification of 100 $\times$ using an Olympus OM-T4 camera (Olympus Corporation, Woodbury, NY, USA).

Results

Expression of connexin-43 in cultured cells

After culture for 4 days, connexin-43 was expressed in the luteal cells at all stages of luteal development examined. Positive immunoreactivity was observed as brown reaction products (Fig. 1a; positive immunoreactive material is dark grey) at the cell periphery. In the absence of the primary antibody, no immunoreactivity was observed (Fig. 1b). Preliminary experiments indicated that maximum connexin-43 expression and progesterone secretion occurred after the cells had been in culture for 4 days. Western blot analysis indicated that the protein is expressed as a doublet at all stages of luteal development with bands identifiable at 43 and 45 kDa. The band at 45 kDa was determined to be in a phosphorylated form, since treatment with alkaline phosphatase of hCG- and OT-treated cells eliminated this band on Western blots. This band is referred to as the band at 43P kDa or connexin-43P (Fig. 2).

Effect of increasing concentrations of hCG and OT on connexin-43 expression

In the early luteal phase cells, hCG at doses 10, 25 and 50 IU/ml increased the total expression (bands at 43 and 43P kDa quantified together) of the protein in a dose-dependent manner (Fig. 3a and b). Thus at 10 IU/ml, hCG increased ($P<0.05$) connexin-43 expression 1.9-fold and at 50 IU by 5-fold as compared with the control. OT also stimulated the total expression of connexin-43, with 50 mU/ml increasing its expression 2.5-fold and 200 mU/ml increasing it 4-fold. A more significant observation is that, in the presence of hCG at the doses examined (10, 25, 50 IU/ml), a statistically significant increase in the non-phosphorylated band and phosphorylated band was observed, compared with non-hCG-treated cells. With 10 IU/ml hCG, a 3-fold increase in the non-phosphorylated band at 43 kDa and a 2-fold increase in the

Figure 1 Immunohistological localization of connexin-43 in cultured luteal cells from the mid-luteal phase baboon corpus luteum. Cells were in culture for 4 days. Connexin-43 is present at the cell periphery (CP) (a) and was also identified in the early and late luteal phase cells (not shown). In the absence of the primary antibody, reaction products were not observed (b). Nuclei (N) were localized with hematoxylin. Scale bar represents 20 μ m. Magnification $\times$ 100.

phosphorylated connexin-43 occurred. At 50 IU/ml hCG a 4-fold increase in both the phosphorylated and non-phosphorylated connexin-43 was observed. In the presence of OT, both connexin-43 and connexin-43P increased in equal proportions, except with 200 mU/ml

OT, when the increase in connexin-43P was greater (4-fold) than the increase in connexin-43.

Effect of hCG and OT on the expression of connexin-43

In untreated cells, maximum expression of total connexin (connexin-43 and connexin-43P) was observed in the mid-luteal phase cells (Fig. 4). Densitometric analysis of the band intensities of three blots show the following distribution: early luteal phase $32 \pm 3\%$, mid-luteal phase $42 \pm 2\%$ and late luteal phase $26 \pm 4\%$. The mid-luteal phase values were statistically significantly different ($P < 0.05$). Maximum expression of connexin-43 was also observed in the mid-luteal phase in tissue homogenates, as shown in previous studies (Khan-Dawood *et al.* 1996b).

hCG stimulated the expression of total connexin-43 in the early, mid- and late luteal phase cells, with maximum effectiveness in the early luteal phase (Figs 5a, 6a and 7a). Also, in the early luteal phase, a significantly greater expression of the non-phosphorylated connexin-43 was observed in comparison with connexin-43P. In contrast, in the mid-luteal phase, the expression of connexin-43P was significantly greater than the non-phosphorylated connexin-43. In the late luteal phase, the stimulatory effect of hCG was significantly lower than in the early and mid-luteal phase, with no significant difference between the phosphorylated and non-phosphorylated connexin-43 (Figs 5b, 6b and 7b).

Figure 2 Representative Western blot of mid-luteal phase corpora lutea protein connexin-43 (Cx43) after treatment of cells with hCG (lanes A and C) and OT (lanes B and D). Bands in lanes C and D show protein before treatment with alkaline phosphatase ($n=3$), and bands in lanes A and B, after treatment with alkaline phosphatase ($n=3$). The phosphorylated band at 45 kDa (43P) is absent from the alkaline phosphatase-treated protein. A 10 μ g sample of cell protein was analyzed in each case.

Figure 3 (a) Representative Western blot of the dose effect of hCG and OT on the expression of connexin-43 (Cx43) in cells obtained from an early luteal phase corpus luteum. Cells were cultured for 4 days. Connexin-43 is expressed as a doublet at 43 and 45 kDa. Connexin-43P is at 45 kDa. A 10 µg sample of cell protein was analyzed in each case. The positions of the prestained molecular mass standards are indicated by the bars on the left. (b) Comparison of the levels of connexin-43 and connexin-43P with increasing concentrations of hCG (10, 25, 50 IU/ml) and OT (50, 100, 200 mU/ml). All data are compared with untreated control cells, which were designated as 100%. Values are means $\pm$ S.E.M. for three experiments. * $P < 0.05$ compared with the control.

OT also had a stimulatory effect on the expression of total connexin-43 in the early and mid-luteal phase cells, with a greater increase in the mid-luteal phase (Figs 5a, 6a and 7a). The OT-stimulated expression of connexin-43 and connexin-43P was similar in the early and mid-luteal phases. Interestingly, in the late luteal phase, the expression of the non-phosphorylated connexin-43 was significantly higher than connexin-43P.

In the presence of hCG (10 IU/ml) and OT (200 mU/ml) together, the expression of total connexin was significantly higher than the control only in the late luteal phase cells. In contrast, in the mid-luteal phase, hCG and OT treatment resulted in a 50% decrease in the total expression of connexin-43. In the early and late luteal phase cells, a significantly higher increase was observed in connexin-43 compared with connexin-43P. The expres-

Figure 4 Representative Western blot of early luteal phase (lane A), mid-luteal phase (lane B) and late luteal phase (lane C) cells cultured for 4 days showing expression of connexin-43 (Cx43). Abundant expression in the mid-luteal phase, which was significantly ($P \leq 0.05$) different from that in the early and late luteal phases, confirms previous *in vivo* data. Lane D is a cardiac tissue control. A 10 µg sample of cell protein was analyzed in each case ($n=3$).

sion of the phosphorylated form in the hCG- and OT-treated early luteal phase cells was not significantly different from the untreated cells. In the mid-luteal phase, a greater ($P < 0.05$) decrease in the non-phosphorylated connexin-43 was observed compared with connexin-43P (Figs 5b, 6b and 7b).

Effect of clomiphene citrate and onapristone on connexin-43 expression in the mid-luteal phase

Both clomiphene citrate (30 nmol/ml) and onapristone (300 nmol/ml) significantly decreased the expression of total connexin-43, with the effect on the phosphorylated and non-phosphorylated forms being similar. However, when hCG was added with clomiphene citrate or onapristone, the total expression of connexin-43 was not affected, compared with the cells treated with clomiphene citrate or onapristone only. When clomiphene citrate and onapristone were combined, the expression of total connexin-43 was also not different from the untreated cells (Fig. 8a and b).

Discussion

The corpus luteum, formed by the cellular components that remain in the follicle after ovulation in response to a mid-cycle surge of pituitary LH, consists of a variety of cell types, which interact closely to perform its major role of synthesizing and secreting progesterone (O'Hara *et al.* 1987, Brannian & Stouffer 1991, Fritz & Fitz 1991, Retamales *et al.* 1994). Two morphologically and biochemically different cells that synthesize progesterone have

Figure 5 (a) Representative Western blot of cells from the early luteal phase corpus luteum (CL) demonstrating the expression of connexin-43 (Cx43) in the presence of hCG (10 IU/ml), OT (200 mU/ml) or hCG+OT (10 IU/ml and 200 mU/ml), and in the absence of hCG and OT (lane C). A 10 µg sample of cell protein was analyzed in each case. The positions of the prestained molecular mass standards are indicated by the bars on the left. (b) Densitometric analysis of the effect of hCG (10 IU/ml) and OT (200 mU/ml) on the expression of non-phosphorylated connexin-43 (band at 43 kDa) and the phosphorylated connexin-43, connexin 43P (band at 45 kDa) in the early luteal phase cells. The value obtained for control cells was designated as 100%. Values are means $\pm$ S.E.M. for three experiments. * P < 0.05 compared with the control.

been identified. In most species examined, one of these cells responds to LH/hCG stimulation via LH/hCG receptors and synthesizes progesterone; the other cell has the capacity to synthesize progesterone in the absence of an LH stimulus, although LH receptors are also present on these cells (Harrison *et al.* 1987, Fritz & Fitz 1991).

Figure 6 (a) Representative Western blot of cells from a mid-luteal phase corpus luteum (CL) demonstrating the expression of connexin-43 (Cx43) in the presence of hCG (10 IU/ml), OT (200 mU/ml) or hCG+OT (10 IU/ml and 200 mU/ml), and in the absence of hCG and OT (lane C). A 10 µg sample of protein was analyzed in each case. The positions of the prestained molecular mass standards are indicated by the bars on the left. (b) Densitometric analysis of the effect of hCG (10 IU/ml) and OT (200 mU/ml) on the expression of connexin-43 (band at 43 kDa) and connexin-43P (band at 45 kDa) in mid-luteal phase cells. The value obtained for control cells was designated as 100%. Values are means $\pm$ S.E.M. for three experiments. * P < 0.05 compared with the control.

In the baboon corpus luteum, we have observed that the absolute concentrations of progesterone secreted by a mixed population of the two steroidogenic cell types (the 'large cell' and the 'small cell') in culture is significantly greater than when the same number of cells are cultured separately (Khan-Dawood *et al.* 1996a). A further observation was that the total concentration of progesterone in the medium was greater when cell-to-cell contact occurred (Khan-Dawood *et al.* 1996a). This evidence

Figure 7 (a) Representative Western blot of cells from a late luteal phase corpus luteum (CL) demonstrating the expression of connexin-43 (Cx43) in the presence of hCG (10 IU/ml), OT (200 mU/ml) or hCG+OT (10 IU/ml and 200 mU/ml), and in the absence of hCG and OT (lane C). A 10 µg sample of protein was analyzed in each case. The positions of the prestained molecular mass standards are indicated by the bars on the left. (b) Densitometric analysis of the effect of hCG (10 IU/ml) and OT (200 mU/ml) on the expression of connexin-43 (band at 43 kDa) and connexin-43P (band at 45 kDa) in late luteal phase cells. Value obtained for control cells was designated as 100%. Values are mean ± S.E.M. for three experiments. *P < 0.05 compared with the control.

suggests that cell-to-cell communication between the two cell types via contact-dependent interaction (Redmer *et al.* 1991, Grazul-Bilska *et al.* 1994) enhances the secretion of progesterone.

Contact-dependent communication between cells involves the formation of several junctions including gap

Figure 8 (a) Representative Western blot of cells from a mid-luteal phase corpus luteum demonstrating the expression of connexin-43 (Cx43) in the presence of hCG (10 IU/ml), OT (200 mU/ml), hCG+OT (10 IU/ml and 200 mU/ml), clomiphene citrate (CC) (30 nmol/ml), CC+hCG (30 nmol/ml+10 IU/ml), onapristone (ZK) (300 nmol/ml), ZK+hCG (300 nmol/ml+10 IU/ml) and ZK+CC (300 nmol/ml+30 nmol/ml). The positions of the prestained molecular mass standards are indicated by the bars on the left. A 10 µg sample of protein was analyzed in each case. (b) Densitometric analysis of the effect of hCG (10 IU/ml), OT (200 mU/ml), CC (30 nmol/ml) and ZK (300 nmol/ml) on the expression of connexin-43 (band at 43 kDa) and connexin-43 P (band at 45 kDa). Value obtained for control cells was designated as 100%. Values are mean ± S.E.M. for three experiments. *P < 0.05 compared with the control.

junctions, tight junctions and adherent junctions (Farquhar & Palade 1963). Gap junctions form transmembrane channels between cells linking the cytoplasm of adjacent cells and through which low molecular mass molecules including second messengers are able to pass (Gilula *et al.* 1972, Loewenstein 1981, Elgang *et al.* 1995). Gap junctions have been identified in corpora lutea of several species by electron microscopy (Adams & Hertig 1969, Abel *et al.* 1975, Albertini & Anderson 1975, McClellan *et al.* 1975, Crisp & Dessouky 1980, O'Shea *et al.* 1990), including the baboon (Khan-Dawood *et al.* 1996b). These junctions consist of a family of homologous proteins, the connexins, which are components of the hexameric connexon. One of these proteins, connexin-43, is present in

ovine, bovine, baboon and human corpora lutea (Grazul-Bilska *et al.* 1994, Khan-Dawood *et al.* 1996b).

Our previous studies have shown that connexin-43 is expressed in both human and baboon corpora lutea, with abundant expression in the mid-luteal phase but undetectable levels in corpora albicantia *in vivo*, suggesting that the protein may be hormonally regulated (Khan-Dawood *et al.* 1996b). The present study confirms these observations when luteal cells from the early, mid- and late luteal phases were cultured *in vitro*. The presence of gap junctions and the associated protein connexin-43 in the corpus luteum suggests that the cells in this tissue are metabolically linked and are capable of cross-communication. The differential expression throughout luteal development further suggests that cell-cell interaction may be regulated (Nnamani *et al.* 1994). Using functional assays involving fluorescence recovery after photobleaching and laser cytometry or after passage of fluorescent Lucifer Yellow after microinjection of luteal cells, it has been shown that luteotrophic/luteolytic hormones and cAMP modulate cell-cell interaction via gap junctions in bovine and ovine corpora lutea (Redmer *et al.* 1991, Grazul-Bilska *et al.* 1994). LH increased the rate of interaction between small and large, and small and small luteal cells but not between large and large cells. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) stimulated the rate of dye transfer between small and large bovine cells and between ovine large and large cells; however, it decreased the rate of dye transfer between ovine small luteal cells. $PGF_{2\alpha}$ also counteracted the stimulatory effect of LH on interaction between ovine large and small cells (Redmer *et al.* 1991, Grazul-Bilska *et al.* 1994, 1996).

The formation of functional gap junctions requires several stages, and for cells to become communication-competent, phosphorylation of the connexins is necessary (Stagg & Fletcher 1990, Beyer 1993). In the rat ovarian follicle, the process of connexin-43 phosphorylation is regulated by the gonadotropins. Thus LH, forskolin and gonadotropin-releasing hormone agonist stimulated both the phosphorylation and dephosphorylation of connexin-43, but the effect of these factors was dependent on the time of exposure of the cells to these factors (Granot & Dekel 1994).

In the baboon and human corpus luteum we have shown that connexin-43 is present as two bands at 43 and 45 kDa (Khan-Dawood *et al.* 1996b). The present study demonstrates that the band at 45 kDa is a phosphorylated protein. Both hCG and OT had modulatory effects on the phosphorylation of connexin-43 in the baboon luteal cells in culture. The effect was dependent on the stage of luteal development. Abundant expression of the phosphorylated protein occurred in the presence of both hCG and OT in the mid-luteal phase cells. This suggests that the corpus luteum cells achieve maximum communication competency with a probable increase in the rate of communication at a time when large quantities of progesterone synthesis occurs. The absence of connexin-43 from the

corpus albicans, which synthesizes little progesterone, supports the contention that gap junction communication is necessary in luteal cells for progesterone synthesis. Loss of communication competency may be the cause of luteolysis. In support of these observations, LH, dibutyryl cAMP and forskolin effectively increased the rate of gap junctional intracellular communication between cells in the mid-luteal phase of the bovine luteal cycle (Grazul-Bilska *et al.* 1996) relative to the early and late luteal phase.

In electrically excitable tissue, gap junction function and connexin-43 expression is regulated by OT (Garfield *et al.* 1990). The direct effect of OT on progesterone synthesis in the corpus luteum has been extensively investigated with no resulting definitive data (Khan-Dawood 1997). The present study suggests that OT may be involved in the expression and phosphorylation of the gap junctional protein in the corpus luteum. Tissue concentrations of OT and OT receptors have been determined to be significantly higher in the mid-luteal phase of the baboon and human corpus luteum (Dawood & Khan-Dawood 1986, Khan-Dawood 1988, Khan-Dawood & Dawood 1993), and a significantly higher stimulatory effect of OT on mid-luteal phase cell connexin-43 expression has been observed in the present studies. Collectively, this suggests that OT may modulate contact-dependent cell-cell communication by stimulating connexin-43 expression.

In contrast with the effect of hCG and OT, both clomiphene citrate, which has anti-estrogen properties (Clark & Markaverich 1982), and onapristone, a progesterone receptor antagonist (Elger *et al.* 1986), decreased the expression of connexin-43 (phosphorylated and non-phosphorylated forms) in the baboon luteal cells. Co-treatment with hCG did not alter the effect. Evidence suggests that estrogen and progesterone regulate the presence of gap junctions (Garfield *et al.* 1990), estradiol stimulates the transcription of connexin-43 (Lye *et al.* 1993, Petrocelli & Lye 1993), while progesterone alone or in combination with estradiol suppresses the expression of connexin mRNA (Grummer *et al.* 1994) in the myometrium and endometrium respectively. The effect of clomiphene citrate is consistent with its anti-estrogen effects. In contrast, the effect of onapristone in suppressing connexin-43 in the corpus luteum is not similar to that observed in other tissues. Only progesterone receptors are present in human (Iwai *et al.* 1990), rhesus monkey (Hild-Petito *et al.* 1988) and baboon (Khan-Dawood *et al.* 1993) corpora lutea, and steroidal regulation of luteal function is suggested (Stouffer & Duffy 1995). However, both estrogen (Yu *et al.* 1994) and progesterone response elements in the connexin-43 gene are recognized (Lye 1993, Yu *et al.* 1994), suggesting direct interaction.

In summary, the present findings further support our previous data (Khan-Dawood *et al.* 1996a, b) and indicate that the expression of connexin-43 in the primate corpus luteum is regulated by both endocrine (LH/hCG) and paracrine/autocrine (OT, estradiol and progesterone)

factors. These factors not only regulate connexin-43 expression but also modulate the phosphorylation of the protein, rendering it active, with differential effects on phosphorylation during luteal development. The presence of the phosphorylated protein suggests communication-competent steroidogenic cells, and therefore it is probable that the LH/hCG-responsive cells may communicate with the LH/hCG-non-responsive cells via gap junctions in the overall synthesis and secretion of progesterone in the corpus luteum.

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