Progesterone Release of Bovine Corpus Luteum in Response to Oxytocin in Different Culture Systems

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Abstract. The aim of the present study was to investigate the effect of oxytocin (OT) on progesterone secretion by bovine corpus luteum (CL) in different culture systems. When luteal cells were cultured for 1, 2, 4 and 15 h at a density of 6×10^4 cells/ml, OT (10^{-7} M) did not affect progesterone secretion. On the other hand, bovine LH strongly stimulated progesterone secretion by the cells 2–15 h after incubation ($P < 0.01$). In another experiment, a novel luteal slice culture system was used, in which luteal cells maintained cell-to-cell contact. Three slices (15–20 mg/a slice) from a CL were pre-incubated for 1 h, and then each slice was incubated for 1 h in a culture tube with a medium (3 ml) supplemented with and without bovine LH (100 ng/ml) or OT (10^{-7} M). Slices were replaced every 1 h in a fresh medium during the 5 h incubation experiment. A 1-h stimulation with bovine LH and OT induced significant but different effects on the release of progesterone. OT acutely stimulated progesterone release at doses 10^{-7} M (155%; $P < 0.05$), and bovine LH significantly increased progesterone release during and after stimulation (223%; $P < 0.01$). These data support the hypothesis that OT plays a role in modulating the mechanisms of progesterone secretion as an intraluteal regulator. Differences observed between the two systems might be due to the presence or absence of cell-to-cell contact.

Key words: Oxytocin, Cell-to-cell contact, Corpus luteum, Bovine.

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It is well known that oxytocin (OT) is synthesized and secreted by the corpus luteum (CL) in many species [1–7]. Moreover, the presence of OT receptors in the CL has been demonstrated in the cow [8], pig [9] and sheep [10]. Although these data suggest that luteal OT plays an important physiological role as an autocrine or paracrine regulator or both, a satisfactory explanation of the physiological role of OT in the CL has not been provided. *In vitro* models, which are mainly based on the incubation of dispersed luteal cells, have shown a variety of CL responses to OT in terms of progesterone production: a luteotropic effect [11],

a luteolytic effect [12], or no effect [13, 14]. These different results obtained from *in vitro* studies seem to depend on the experimental model, e.g., short- or long-term culture of luteal cells, cell density and duration of stimulation with reagents.

Recently, it has been reported that the communication between large and small luteal cells is very important for the function of the CL [15]. In addition, Miyamoto *et al.* [16] reported that OT stimulated progesterone production from bovine CL using an *in vitro* microdialysis system (MDS). In contrast to classical cell culture techniques, MDS maintains cell-to-cell contact as is the case *in vivo*. Thus, it is strongly suggested that cell-to-cell contact may be a requisite for the CL response to OT.

The present study was conducted to investigate

the effect of OT on progesterone secretion by the bovine corpora lutea *in vitro* under different culture conditions with and without cell-to-cell contact.

Materials and Methods

Luteal cell culture

Ovaries with a CL were collected from Holstein cows at a local slaughterhouse within 10–20 min after exsanguination. The luteal phase was classified by macroscopic observation of the ovary as described previously [17]. Only those CLs assigned to the mid-luteal phase (Days 8–12 after ovulation) were collected. The ovaries were submerged in ice-cold physiological saline before being transported to the laboratory. Luteal tissue dissociation and cell preparation were performed as previously described by Okuda *et al.* [8]. After perfusion with ice-cold EGTA buffer (0.1 mM EGTA, 10 mM HEPES, 140 mM NaCl, 7.1 mM KCl; pH 7.4) for 15 min to remove vascular blood and to loosen the connection between the vascular endothelial cells, the CL was subsequently perfused with washing buffer (10 mM HEPES, 140 mM NaCl, 7.1 mM KCl, 5.0 mM CaCl_2 ; pH 7.4) for 15 min. Final dissociation was obtained by perfusion for 30 min with the washing buffer containing 0.05% collagenase (Sigma, C0130) and 0.1% bovine serum albumin (BSA; Boehringer Mannheim GmbH, 735078). All perfusion buffers were gassed with 5% CO_2 in 95% O_2 during perfusion. Luteal cells were dispersed from the CL matrix with steel combs. Dissociated CL cells were pooled and stirred for 30 min in Dulbecco's Modified Eagle's medium (DME; Sigma Chemical Co., St. Louis, MO, D1152) containing 0.05% collagenase, 0.005% Deoxyribonuclease I (DNase I; Sigma, D5025), and 0.1% BSA, and then filtered through metal meshes (200 μm , 150 μm , and 80 μm) to remove undissociated tissue fragments. The filtrate was washed three times by centrifugation for 10 min at $50 \times g$ with the culture medium, Dulbecco's Modified Eagle's Medium and Ham's F-12 medium, 1:1 (vol:vol; DMEM/F-12; Sigma, D8900) supplemented with 10% calf serum (CS; Sigma, C6278) and 20 $\mu\text{g}/\text{ml}$ gentamicin. The cells were counted with a hemocytometer. Cell viability was higher than 75% as assessed by trypan blue exclusion. The obtained cell suspension contained very few endothelial cells or fibrocytes (0–10%), and no erythrocytes. The dispersed cells were seed-

ed at 6×10^4 cells/ml viable cells, in 48-well culture plates (Costar, Cambridge, MA, USA) and cultured for 39 h in humidified atmosphere of 5% CO_2 in air at 37.5 C. Culture media were replaced at 24 h with a fresh medium containing the following stimulators: 10 ng/ml bovine LH (USDA-bLH-B-6) and 10^{-7} M OT (Teikoku Hormone MFG. Co., Tokyo, Japan). After 1, 2, 4 or 15 h incubation, conditioned media were collected and stored at -35°C until assayed for progesterone.

Luteal slices preparation

The experimental procedure using sliced tissue is shown in Fig. 1. CL of the mid-luteal phase

Fig. 1. Schematicgraph of the experimental procedure for luteal slice culture.

(Day 8–12) were sliced in a layer approximately 0.3 mm thick with a tissue slicer (NATUME Co., Ltd. Tokyo) and trimmed into 1 cm² pieces (15–20 mg). Three slices were made from one CL. After hanging the slices with steel needles (KATSUICHI Co., Ltd.), the slices were pre-incubated with DMEM/F-12 containing 0.5% CS in a culture tube (12 × 75 mm) for 1 h. Each slice was stimulated with bovine LH (100 ng/ml) and OT (10⁻⁷ M) for 1 h shaking gently at 37.5 C, and for the next 4 h, each slice was transferred every hour into a fresh medium (3 ml). The media were continuously gassed with 5% CO₂/air during incubation. Before replacing the medium, each slice was washed three times with fresh medium and blotted with absorbent paper. After incubation, the collected media were frozen at -35 C until the progesterone assays.

Hormone determination

Concentrations of progesterone were determined directly from the cell culture media with an enzyme immunoassay [18]. Progesterone 3-(O-carboxymethyl) oxime linked to peroxidase type VI through a 6-aminocaproic acid spacer (Sigma, P3659) was used as a tracer (1:120,000 final dilution). Antiserum of progesterone (OK-1) was produced in our Laboratory in a female rabbit (New Zealand White) by immunization against 11 α -hydroxy progesterone-11-hemisuccinate-BSA (Sigma, H4508). Cross-reactivities of anti-progesterone serum, which were validated by comparing the inhibition of binding of peroxidase-labeled progesterone to antiserum, were 100% progesterone, 7.1% 5 α -pregnanedione, 2.4% pregnenolone, 1.2% deoxycorticosterone, less than 1.0% for 17 α -hydroxy-progesterone, 20 α -hydroxy-progesterone, corticosterone, estradiol-17 α , testosterone and androstenedione. The final antibody dilution was 1:600,000. The standard curve ranged from 1.27 to 318 nM and the effective dose for 50% inhibition (ED₅₀) of the assay was 30.4 nM. The intra- and inter-assay coefficients of variation were 5.4% and 8.6%, respectively.

Statistical analysis

The data are shown as the mean $\pm$ SEM of values obtained in the separate experiments, each performed in triplicate. For the statistical analyses, the relative percentages of the control value were used. The statistical significance of differ-

ences between control or bLH-treated groups and the OT-treated groups was assessed by means of analysis of variance followed by Duncan's new multiple range test.

Results

Effect of OT on progesterone secretion by cultured bovine luteal cells

Figure 2 shows the time-dependent effects of OT and LH on progesterone production by cultured bovine luteal cells. OT (10⁻⁷ M) had no effect on progesterone secretion by luteal cells regardless of the incubation time. On the other hand, bovine LH significantly stimulated progesterone secretion by cultured bovine luteal cells independent of stimulation time ($P < 0.01$).

Effect of LH on progesterone secretion by cultured bovine luteal tissue

The progesterone secretion by CL slices is shown

Fig. 2. Time-dependent effect of OT (10⁻⁷ M) on progesterone secretion by bovine luteal cells seeded at 6×10^4 cells/ml. Cells were cultured for a total of 39 h, and culture media were replaced at 24 h. Progesterone levels in the last 15 h of culture were determined. Data are expressed as the mean $\pm$ SEM of four separate experiments, each performed in triplicate. After stimulation for 15 h, the basal progesterone concentration secreted into the medium was 500 ± 75 ng/ml. Different superscripts indicate significant differences ($P < 0.05$ or lower), as determined by analysis of variance followed by Duncan's new multiple range test.

in Fig. 3. The release of progesterone from unstimulated luteal slices did not change during the entire incubation period. On the other hand, LH significantly stimulated the progesterone secretion by CL slices from the start of exposure until the end of incubation ($P<0.01$).

Effect of OT on progesterone secretion by bovine luteal tissue

After stimulation of the slices by 10^{-7} M OT, progesterone secretion increased up to 155% compared to the progesterone release in the first 60 min ($P<0.05$). After OT stimulation, the progesterone release rapidly decreased to the control levels. In contrast, LH significantly stimulated the progesterone release from the start of exposure until the end of incubation ($P<0.01$).

Discussion

The present study clearly shows that OT stimulated progesterone secretion by bovine luteal slices, although the above action of the reagent was not observed in a cell culture system.

Earlier culture studies, which were carried out at cell densities of 1×10^4 – 1×10^5 cells/ml, reported an absence of effect of OT on progesterone production by the luteal cells; i.e. dispersed human luteal cells from the early, mid- and late luteal phases cultured at a density of 3×10^4 cells/ml did not affect progesterone production, in spite of the presence of a high concentration of OT (10^{-6} M) for 2 h [19]. Likewise, bovine luteal cells from Days 4–18 of the estrous cycle (1×10^5 cells/ml), when incubated with OT (400 mIU/ml) for 2 h did not show increased progesterone production [20]. The present study demonstrated a significant effect of OT on the progesterone production by bovine luteal tissues, in which cell-to-cell contact seems to be maintained. Our results suggest that cell-to-cell contact is an important factor for stimulating progesterone production in bovine CL with OT *in vitro*. These results are in agreement with those of Miyamoto *et al.* [16] who reported that progesterone production by microdialyzed bovine CL was stimulated by administration of OT. The lack of effect of OT on progesterone production by luteal cells *in vitro* seems to be the result of inadequate cell-to-cell communication, and further studies are required to clarify these points.

Fig. 3. Effect of LH on progesterone secretion by bovine luteal tissue. LH was administered between 0 and 60 min. Bars represent mean $\pm$ SEM of five separate experiments. Asterisks indicate values significantly higher than progesterone secretion for pre-incubation (* $P<0.05$, ** $P<0.01$), as determined by analysis of variance followed by Duncan's new multiple range test.

Fig. 4. Effects of OT and bovine LH on progesterone secretion by cultured bovine luteal tissue. Luteal slices were incubated for a total of 300 min, and stimulators were added between 0 and 60 min after the start of culture (). The basal progesterone secretion after pre-incubation was used as the control (100%) (). Data are expressed as the mean $\pm$ SEM of five experiments. Asterisks indicate values significantly higher than the control (* $P<0.05$, ** $P<0.01$), as determined by analysis of variance followed by Duncan's new multiple range test.

In the present study, LH rapidly stimulated progesterone production by both cultured luteal cells and luteal slices; and, in the luteal slice culture system, OT acutely stimulated progesterone production. It is generally accepted that the stimulatory effect of LH on progesterone production is mediated via specific LH receptors that are mostly in the small luteal cells. LH stimulates progesterone secretion from small cells via the cAMP/PK-A pathway, but has little effect on large cells. We have provided evidence for OT receptors in bovine granulosa cells [21], and suggested that the large luteal cells may possess OT receptors. Thus, it is hypothesized that the first target cells for OT might be large luteal cells, and that some substances produced in these cells might stimulate progesterone secretion by small luteal cells via cell-to-cell contact. This cell-to-cell contact may be intact and functional in luteal slices. However, in the cell culture system, cell-to-cell contact does not seem to be fully functional, so the luteotropic effect of OT might not be observed.

OT (10^{-7} M) significantly stimulated progesterone production by bovine luteal tissues. Since tissue concentrations of OT in CL of the mid-luteal phase (Day 8–12) were within the range of 0.1–1 $\mu\text{g/g}$ wet weight [22], the dose of OT (10^{-7} M) used in this study seems to be of a physiological concentration. However, the stimulatory effect of OT did not appear in cell culture system, and one possible explanation for this discrepancy is that the accessory cells (e.g., fibroblasts and endothelial cells) may have a physiological role in CL function, since luteal cells were contaminated with few accessory cells in our cell preparation technique. It has been reported that endothelial cells constitute the major proportion (53.5%) of CL cells [23, 24], and Girsh *et al.* [25] reported that progesterone production by luteinized bovine granulosa and theca cells was modulated when co-cultured with

endothelial cells. Furthermore, endothelial cells secrete prostaglandin I₂ [26] and endothelin-1 [27], which have been reported to affect steroidogenesis [28–30]. These data suggest that the luteotropic effect of OT might be modulated by unknown factors produced by nonsteroidogenic cells in this tissue culture system. Further study is needed to clarify the physiological roles of the accessory cells (e.g., fibroblasts and endothelial cells) and their products in CL function.

In summary, the present study demonstrates the luteotropic actions of OT when luteal slices are stimulated with the reagent. However, the above action of OT was not observed in the cell culture system. The different response between the two *in vitro* culture systems we used might be caused by factors such as cell-to-cell contact, and communication within luteal cells and with other cells, which may be essential for the response of the CL to OT, as well as the time of peptide stimulation. Based on these results, culture conditions should be considered when testing the effects of a reagent on the function of an organ *in vitro*.

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