Binding and action of insulin-like growth factors and insulin in bovine luteal tissue during the oestrous cycle

H. Sauerwein, A. Miyamoto, J. Günther, H. H. D. Meyer and D. Schams*

*Institut für Physiologie, Süddeutsche Versuchs und Forschungsanstalt für Milchwirtschaft,
Technische Universität München, D-8050 Freising-Weihenstephan, Germany*

Summary. The effect of insulin-like growth factors (IGFs) and insulin on the release of progesterone and oxytocin from bovine corpus luteum was investigated at early (days 5–7), mid- (days 8–12) and late (days 15–18) luteal phases of the oestrous cycle in an *in vitro* microdialysis system. The expression of specific receptors was evaluated in bovine corpora lutea of the respective luteal stages.

A 30 min infusion of IGF-1, IGF-2 (1.3, 13 and 130 nmol l⁻¹) or insulin (13, 130 and 1300 nmol l⁻¹) caused a stimulation of the release of progesterone ($P < 0.05$). IGF-1 was most effective in releasing progesterone. Oxytocin release from corpora lutea was stimulated by insulin at all doses tested (13–1300 nmol l⁻¹), whereas the IGFs were only effective at the highest dose (130 nmol l⁻¹) applied. The high doses of IGFs (130 nmol l⁻¹) and insulin (1300 nmol l⁻¹) stimulated the release of progesterone and oxytocin throughout the luteal phase ($P < 0.05$). For all three peptides, greatest stimulation was seen during the late luteal phase (days 15–18 of the oestrous cycle) with the peak of progesterone release directly related to peptide infusion ($P < 0.05$). In addition, IGF-1 stimulated total release of progesterone (units in 4 h) after the beginning of the stimulation during this phase ($P < 0.05$). IGF-1 caused a gradual increase of progesterone even beyond the time of peptide perfusion, whereas IGF-2 and insulin stimulated progesterone release only during the peptide perfusion.

Distinct receptors for IGF-1 and IGF-2 were present in corpora lutea membrane preparations at all stages investigated. Specific binding for insulin was also seen in all stages of the cycle without any cycle-dependent changes in the amount of binding. The displacement of labelled insulin by unlabelled IGF-1 and IGF-2 did not show the rank of order that has been described as typical for insulin receptors (i.e. insulin > IGF-1 > IGF-2), but comparable binding affinities were observed for the three unlabelled ligands. Specific binding of IGF-2 was markedly higher than that of IGF-1 or insulin throughout the cycle (1.9- and 4.9-fold higher compared with IGF-1 and insulin, respectively). Receptor specificity did not change during luteal development. Binding affinity and capacity of IGF-1 receptor was constant throughout the oestrous cycle. Specific IGF-2 binding increased and showed a positive co-operativity towards the end of the cycle. Specific binding of insulin was not significantly different in the three luteal stages examined.

Keywords: corpus luteum; IGFs; insulin; progesterone; oxytocin; cow

Introduction

Insulin-like growth factor 1 (IGF-1) is a basic peptide of 70 amino acids which stimulates a variety of biological processes (Rinderknecht & Humbel, 1978). The granulosa cells are sites of its production (Hammond *et al.*, 1985; Davoren & Hsueh, 1986), reception (Veldhuis *et al.*, 1985; Davoren

*Reprint requests.

et al., 1986; Adashi *et al.*, 1986) and action (Savion *et al.*, 1981; Adashi *et al.*, 1984; Davoren *et al.*, 1985; Schams *et al.*, 1987). Bovine corpus luteum was also defined as a site of IGF-1 mRNA expression, peptide production and its action (Einspanier *et al.*, 1990). Both chronic and acute stimulation of the two major hormone products of bovine corpus luteum, progesterone and oxytocin by IGF-1 led to speculation that IGF-1 may be an autocrine/paracrine factor in cows (McArdle & Holtorf, 1989; Einspanier *et al.*, 1990). If this hypothesis is correct there must be binding sites of IGF-1 in bovine corpora lutea (for review see Rechler, 1988).

The aim of this study was to demonstrate and characterize the binding sites for IGF-1 in bovine luteal tissue. The binding and effects of the related peptides, IGF-2 and insulin, were also investigated. Since expression of IGF-1 mRNA and production of IGF-1 peptide paralleled each other and their activity changed during the oestrous cycle (Einspanier *et al.*, 1990), we investigated how binding of growth factors is related to stimulation of progesterone and oxytocin during the oestrous cycle in cows.

Materials and Methods

Collection of corpora lutea

The corpora lutea from German Fleckvieh cows were collected from a local slaughterhouse within 10–20 min of slaughter. The stage of the cycle was defined by macroscopic observation of the ovaries (follicles and corpora lutea) and the uterus (size, colour, consistency, connective tissue and mucus). The corpora lutea were assigned to stages of the luteal phase, days 5–7, days 8–12 and days 15–18 after ovulation. Corpora lutea from 114 cows were used for all the experiments presented herein. For the *in vitro* perfusion study, the corpora lutea were washed with saline at 38°C several times, and transported to the laboratory in Medium 199 (Sigma, Munich, Germany) at pH 7.2, 38°C containing 10 mmol NaHCO₃ l⁻¹, Earle's salts, L-glutamine, 25 mmol Hepes l⁻¹, 0.5% (w/v) bovine serum albumin, 60 mg penicillin l⁻¹, 100 mg streptomycin l⁻¹, 2 mg amphotericin l⁻¹ and 56 mg ascorbic acid l⁻¹ (M199). For the preparation of microsomal membranes, the corpora lutea were separated from the ovaries immediately, snap frozen in liquid nitrogen and were then stored at -80°C until processed.

Microdialysis perfusion system *in vitro*

The microdialysis perfusion system of Jarry *et al.* (1987) was used with modifications for the *in vitro* organ culture described in detail by Miyamoto & Schams (1991). All manipulations were conducted at 38°C in M199. Each corpus luteum was cut to a 10–12 mm cube, and bisected through the face of the apex (days 8–12 and 15–18) or used without bisection (days 5–7). Each luteal piece was penetrated by an 8–10 mm dialysis capillary (Fresenius SPS 900 Hollow Fibers, Fresenius AG, St Wendel, Germany; cut off M_r 100 kDa, 0.2 mm diameter). The capillary was attached with each end to Silastic tubing (i.d. 0.3 mm) by glue. The tubing was fixed on the face of the luteal piece by Histoacryl blue (B. Braun-Dexon GmbH, Spangenberg, Germany) to hold the dialysis capillary in the luteal tissue. For the perfusion one of the tubes was connected to a peristaltic pump, and the other was joined to the fraction collector. The luteal pieces prepared in this way were maintained in organ culture chambers (2070 Tube, Falcon, USA) filled with 50 ml M199 at 38°C in a water bath. M199 was continuously exchanged at a flow rate of 15 ml h⁻¹. The luteal pieces were perfused by Ringer's solution at a flow rate of 1 ml in 10 min for 8 h; in earlier experiments we demonstrated that the endocrine function of the pieces of corpus luteum, i.e. the progesterone release and its responsiveness to luteinizing hormone (LH) stimuli, is not adversely affected even after 7 h incubation (Miyamoto & Schams, 1991). After 3 h preincubation, perfusate fractions were collected every 10 min for 5 h. Because of the great individual variation of the basal hormone concentrations between luteal pieces, the mean concentrations of the first six fractions (1 h perfusion) were used for calculating an individual progesterone baseline. For oxytocin the baseline was determined as the concentration of the pooled first six fractions. All hormone concentrations in the fractions collected from each piece of corpus luteum were then expressed as a percentage of individual baseline (100%). IGF-1 and IGF-2 were dissolved in acetic acid (10 mmol l⁻¹) to a concentration of 10 mg ml⁻¹ and were then diluted with water to 1 mg ml⁻¹ stock solutions. The IGF solutions were stored at -20°C. Insulin was dissolved in exactly the same way as IGFs just before the start of the experiment. All peptides were diluted with Ringer's solution to the final concentrations needed (for IGFs: range 10–1000 ng ml⁻¹, i.e. 1.3–130 nmol l⁻¹; for insulin: 76–7600 ng ml⁻¹, i.e. 13–1300 nmol l⁻¹). The possibility that the pH of the infusate may produce some artefact effect on the hormone release was excluded by running 1 µmol l⁻¹ (for IGFs) or 7.6 µmol l⁻¹ (for insulin) acetic acid in Ringer's solution in the controls parallel to the various treatments. Effluent fractions were stored at -20°C until assayed for progesterone and oxytocin. The diffusion capacity of the membrane for IGFs and insulin was about 0.1% of the concentration infused as determined in previous methodological experiments.

Progesterone and oxytocin determination

Concentrations of progesterone were determined directly in duplicate 20 μ l samples of the perfusate fractions with an enzyme immunoassay in microtitre plates based on the double antibody technique of Prakash *et al.* (1987). Progesterone-3-(*O*-carboxymethyl)oxime linked to peroxidase through a 6-aminocaproic acid spacer (Sigma, Munich, Germany) was applied as tracer; progesterone-11 α -hemisuccinate-BSA has been used for the production of the antibody that was characterized earlier (Hoffmann *et al.*, 1973). The final antibody dilution was 1:500 000. The standard curve ranged from 0.4 to 100 ng ml⁻¹ and the ED₅₀ of the assay was 10 ng ml⁻¹. The intra- and interassay coefficients of variations were below 10.0%.

Oxytocin concentrations in the pooled fractions (fraction 1–6, 7–9 and 10–15) were directly quantified by radioimmunoassay (Schams, 1983). The standard curves ranged from 0.625 to 80 pg ml⁻¹ and the ED₅₀ of the assay was 14.2 pg ml⁻¹. The intra- and interassay coefficients of variation were 8.7% and 7.8%, respectively.

Membrane preparation

Corpora lutea were transferred into three volumes of 10 mmol Tris l⁻¹, 250 mmol sucrose l⁻¹, 1 mmol CaCl₂ l⁻¹ pH 7.5 and were homogenized in an ice bath with Ultra Turrax equipment (Janke and Kunkel, Staufen, Germany). Five bursts of 15 s at maximum speed with 45 s intervals of cooling between each burst were used. From each cyclic stage three pools each consisting of 6 corpora lutea were prepared. The homogenate was subsequently centrifuged at 800 *g* for 15 min at 4°C. The supernatant was collected and centrifuged at 100 000 *g* for 60 min at 4°C. The pellet was resuspended in 10 mmol Tris l⁻¹, 5 mmol CaCl₂ l⁻¹, 75 mmol NaCl l⁻¹, pH 7.5 and then repelleted. After washing twice, the final pellet was resuspended in the same buffer to a concentration of 250 mg original tissue weight per ml preparation. The preparation was aliquoted and stored at -30°C until assayed.

Radioreceptor assays

Recombinant human IGF-1 (Ciba, Basel, Switzerland), human IGF-2 (Ciba) and insulin (synthetic bovine insulin, Novo, Copenhagen, Denmark) were iodinated by a modified lactoperoxidase method (Thorell & Johansson, 1971) to specific activities of 40–60 μ Ci μ g⁻¹. The receptor preparations were thawed at room temperature and were then added in brief succession to the test tubes containing ¹²⁵I-labelled ligand and unlabelled IGF-1, IGF-2 or insulin. All reagents were prepared in 10 mmol Tris l⁻¹, 5 mmol CaCl₂ l⁻¹, 75 mmol NaCl l⁻¹ pH 7.5, 0.5% (w/v) bovine serum albumin. The assays were performed in duplicate with membrane homogenates of 25 mg corpora lutea weight equivalents per tube containing approximately 300 μ g protein as measured with the BCA method (Smith *et al.*, 1985). Preliminary experiments have shown that the binding of the ¹²⁵I-labels is proportional to the amount of membrane preparation added in the range of membrane concentration used for the present studies. The optimal assay conditions for ¹²⁵I-labelled IGF-1, ¹²⁵I-labelled IGF-2 and ¹²⁵I-labelled insulin binding were first defined for further characterization of the binding sites. Maximum binding was obtained at 4°C after 4 h and remained stable for about 30 h. Because of these findings all subsequent binding experiments were performed at 4°C overnight.

Approximately 0.02 μ Ci ¹²⁵I-labelled ligand (IGF-1, IGF-2 or insulin) was incubated in the presence of 0–10 000 ng unlabelled IGF-1, IGF-2 or insulin per tube for 16 h at 4°C. Equilibrium was reached under these conditions; no significant tracer degradation was observed during the incubation as determined by TCA precipitation (Banskota *et al.*, 1986). The incubation was terminated by addition of 2 ml ice cold 10 mmol Tris l⁻¹, 5 mmol CaCl₂ l⁻¹, 75 mmol NaCl l⁻¹ pH 7.5; bound and free tracer were separated by centrifugation at 3000 *g* at 4°C for 30 min. The reproducibility of the receptor assays were estimated by linear regression analysis of repeated determinations of the binding characteristics. The association constant K_d and the binding capacity B_{max} of all receptor assays taken together showed excellent correlation ($r = 0.990$, slope 1.18 for K_d and $r = 0.873$, slope 1.04 for B_{max}).

Statistical analysis

For the analysis of the microdialysis perfusion system stimulation experiments, the concentrations of progesterone (%) in the first six fractions from each experimental group were pooled to obtain baseline values. The peaks of progesterone or oxytocin release, and the areas under the curves of progesterone release (units in 4 h) after the beginning of the 30 min stimulation by IGFs or insulin at different doses or at different luteal phases of the oestrous cycle were tested by analysis of variance and Duncan's new multiple range test, Student's *t* test or the Cochran-Cox test.

The data obtained on binding of IGF-1, IGF-2 and insulin to membranes of corpora lutea were analysed with the LIGAND programme (Munson & Rodbard, 1980) using nonlinear iterative curve-fitting procedures (McPherson, 1985). The initial parameters were calculated by Scatchard analysis (Scatchard, 1949) and were then iteratively refined until the weighted sum of squares was minimized. The goodness of fit for the selected model was assayed by the 'runs test'. Different models (one or two binding sites) were compared using *F* test statistics to determine whether a change in the model resulted in a significant reduction in the weighted sum of squares. The criteria for rejecting or accepting a particular model were based on the calculated probability values (Munson & Rodbard, 1980).

The final parameter estimates obtained from three pools of three different stages of the cycle were analysed by analysis of variance. The significance of differences between the different cyclic stages were then assessed by Student's *t* test using the Bonferroni correlation for multiple comparison. Data are expressed as means $\pm$ SD.

Results

Effect of various doses of IGF-1, IGF-2 and insulin on the release of progesterone and oxytocin from luteal tissue

A 30 min perfusion with IGF-1, IGF-2 ($1.3\text{--}130\text{ nmol l}^{-1}$) or insulin ($13\text{--}1300\text{ nmol l}^{-1}$) through corpora lutea from days 8 to 12 induced a significant peak of progesterone release ($P < 0.05$; Fig. 1). Comparing the total release of progesterone during 4 h after the beginning of stimulation with the different peptides (i.e. the areas under the curves) the stimulation of progesterone release by IGF-1 was greater than that produced by IGF-2 or insulin ($P < 0.05$; Table 1).

In contrast to the progesterone stimulation, higher IGF concentrations (130 nmol l^{-1}) were needed to stimulate oxytocin release ($146.3 \pm 10.3\%$ for IGF-1 and $139.1 \pm 4.9\%$ for IGF-2, respectively, $P < 0.05$). Insulin showed a semi-dose-dependent effect on oxytocin release ($131.6 \pm 10.5\%$ for 13 nmol l^{-1} , $142.0 \pm 13.1\%$ for 130 nmol l^{-1} and $329.8 \pm 47.8\%$ for 1300 nmol l^{-1} ; $P < 0.05$).

Effect of IGF-1, IGF-2 and insulin on the release of progesterone and oxytocin from luteal tissue at different stages of the luteal phase

IGF-1 (130 nmol l^{-1}), IGF-2 (130 nmol l^{-1}) or insulin (1300 nmol l^{-1}) stimulated the release of progesterone throughout the luteal phase ($P < 0.05$; Fig. 2). All three substances stimulated the peak height of progesterone release during the late luteal phase (days 15–18; $P < 0.05$). Additionally, the effect of IGF-1 on the total release of progesterone (units in 4 h) was greatest after the beginning of the stimulation during this stage ($P < 0.05$; Table 2).

All three peptides stimulated the release of oxytocin throughout the luteal phases ($P < 0.05$; Fig. 3). Insulin was more effective at early to mid-luteal stages than at late luteal phase ($P < 0.05$).

Competition of binding to IGF-1 and IGF-2 receptors

^{125}I -labelled IGF-1 binding was displaced in a dose-dependent manner by unlabelled IGF-1 (Fig. 4). About 80% of ^{125}I -labelled IGF-1 bound to membranes of corpora lutea could be displaced by the highest concentration of unlabelled IGF-1 indicating that nonspecific binding contributed to about 20% of the total binding of IGF-1 to membranes of corpora lutea. IGF-2 was about five times less effective in displacing ^{125}I -labelled IGF-1. Unlabelled insulin showed significant displacement only at concentrations higher than $2\text{ }\mu\text{mol l}^{-1}$.

Specific binding of ^{125}I -labelled IGF-2 to bovine corpora lutea membranes was markedly higher than for ^{125}I -labelled IGF-1 ($39.4 \pm 7.8\%$ versus $20.8 \pm 2.1\%$). Nonspecific binding accounted for 27% of total binding. ^{125}I -labelled IGF-2 was displaced by IGF-2 in a dose-dependent manner whereas IGF-1 and insulin exhibited negligible binding affinities. The specific binding of ^{125}I -labelled insulin ($8.0 \pm 1.1\%$) was lower than that of ^{125}I -labelled IGF-1 or ^{125}I -labelled IGF-2. The binding in the presence of the highest concentration of unlabelled insulin was 36% of total binding. ^{125}I -labelled insulin was displaced by insulin in a dose-dependent manner. IGF-1 and IGF-2 showed comparable binding affinities.

Scatchard plots for the competitive binding of ^{125}I -labelled IGF-1 and unlabelled IGF-1 were linear (Fig. 5), thus indicating a single class of high-affinity binding sites ($K_a =$

Fig. 1. Dose-dependent release of progesterone by (a) insulin-like growth factor (IGF)-1, (b) IGF-2 and (c) insulin from microdialysed bovine corpora lutea *in vitro* (days 8–12 of the oestrous cycle). All values are shown as percentage of the respective baselines (mean $\pm$ SEM; $n = 5$ corpora lutea per group) that were calculated from the first six fractions (perfused with Ringer's solution only). The filled bars indicate the peptide application. Absolute concentrations of progesterone from baselines were 7.31 ± 0.28 ng ml⁻¹ ($n = 60$, mean $\pm$ SEM).

$1.53 \times 10^9 \pm 7.65 \times 10^7$ mol⁻¹, $B_{\max} = 2.07 \times 10^{-10} \pm 5.38 \times 10^{-12}$ mol l⁻¹). In comparison the Scatchard plots of specific IGF-2 binding (Fig. 6) had a markedly higher capacity ($B_{\max} = 2.18 \times 10^{-9} \pm 1.29 \times 10^{-10}$ mol l⁻¹); the affinity was lower ($K_d = 3.49 \times 10^8 \pm 4.78 \times 10^7$ mol⁻¹ l). Scatchard analysis of the insulin binding data showed curvilinear plots (Fig. 5). A two-site model was superior for describing the binding characteristics as demonstrated with the LIGAND program. The affinity constant and the capacity of the high affinity binding site were $K_{d1} = 1.15 \times 10^9 \pm 2.80 \times 10^8$ mol⁻¹ l and $B_{\max1} = 6.70 \times 10^{-11} \pm 1.49 \times 10^{-11}$ mol l⁻¹. For the low affinity site the K_{d2} was calculated as $1.86 \times 10^7 \pm 7.05 \times 10^6$ mol⁻¹ l and the $B_{\max2}$ as $1.08 \times 10^{-9} \pm 2.49 \times 10^{-10}$ mol l⁻¹.

Table 1. Comparison of the areas under the curves of release of progesterone (units in 4 h) after the beginning of the stimulation for 30 min with different doses of insulin-like growth factor (IGF)-1, IGF-2 or insulin in bovine corpora lutea (*n* = 5; mean ± SEM)

	Peptide concentration (nmol l ⁻¹)				
	Control	1·3	13	130	1300
IGF-1	2468 ± 51	2843 ± 179	3185 ± 133***	3123 ± 65***	
IGF-2	2457 ± 28	2726 ± 65**	2666 ± 98 ^b	2719 ± 89 ^{ab}	
Insulin	2409 ± 49		2697 ± 165 ^b	2769 ± 58 ^{ab}	3144 ± 163*

P* < 0·05, *P* < 0·01 and ****P* < 0·001 versus control.
^{a,b}Values with different superscripts in each column differ (*P* < 0·05).

IGF-1 and IGF-2 binding to membranes of corpora lutea during the oestrous cycle

The characteristics of the binding of IGF-1, i.e. *K_a* and *B_{max}*, to membranes of corpora lutea prepared from early (days 5–7), mid- (days 8–12) and late (days 15–18) luteal stages showed no difference when expressed on a per gram original wet weight basis. Displacement studies of ¹²⁵I-labelled IGF-1 with unlabelled IGF-1, IGF-2 and insulin showed the same relative affinities during early and late luteal phase as demonstrated above for the mid-luteal phase (Fig. 5). However, significantly less protein was extracted from corpora lutea of the early luteal phase compared with the mid- and late phases which yielded identical protein concentrations in the membrane preparations. In the late luteal phase unlabelled IGF-2 at low concentrations (0·1–10 ng per tube) augmented ¹²⁵I-labelled IGF-2 binding as much as 40% over that observed in the absence of unlabelled hormone (Fig. 6). This augmentation of binding was minimal or absent in early and mid-luteal phase corpora lutea. No cycle dependent difference was seen in the negligible binding of IGF-1 and insulin to IGF-2 binding sites. Insulin binding characteristics showed no difference in *K_{a1}*, *K_{a2}*, *B_{max1}* and *B_{max2}* between days 5 and 7 and days 8 and 12. High-affinity sites could not be detected in corpora lutea membranes from days 15–18. There was no cycle dependent difference in the affinity of IGF-2 to ¹²⁵I-labelled insulin binding sites. For IGF-1 a relatively higher affinity for insulin binding sites was observed during mid- and late luteal phases when compared with the early stages.

Discussion

In addition to the well-documented effects of IGFs and of insulin on mitogenesis and cell differentiation of bovine granulosa cells (Savion *et al.*, 1981), our study provides further evidence for stimulation by these peptides of secretory processes in bovine luteal tissue. IGFs and insulin stimulated the release of progesterone and oxytocin in the microdialysis perfusion system. Although the mechanism of this acute release of hormones is not clear, comparable acute intracellular events have previously been demonstrated in different systems, e.g. in cultured porcine thyroid cells a very rapid effect (within 1–2 min) of IGF-1 on cytoplasmic pH has been described (Takasu *et al.*, 1990); in mouse adipose tissue an activating effect of insulin on phosphatidylinositol phospholipase C was evident within 5 min (Chou *et al.*, 1990). The acute stimulation of hormone release in our experiments therefore appears as a physiological response of luteal tissue that is possibly based on similar intracellular effects. The question of whether the increased progesterone release results from stimulation of steroid synthesis or whether it reflects an increased release of already synthesized progesterone remains open. The increased baseline that is seen under IGF-1 stimulation might support the concept of an effect on steroidogenesis itself. IGF-1 was the effective factor in stimulating the release of progesterone in the microdialysis perfusion system. The predominant feature of the IGF-1 effect was a gradual increase in progesterone release after the first peak that was directly related to the peptide infusion. In consequence the total release of

Fig. 2. Effect of intraluteal application of (a) insulin-like growth factor (IGF)-1 (130 nmol l^{-1}), (b) IGF-2 (130 nmol l^{-1}) and (c) insulin (1300 nmol l^{-1}) on the release of progesterone at different stages of the luteal phase *in vitro* (mean $\pm$ SEM; $n = 5$ corpora lutea per group). The filled bars indicate the peptide application. Absolute concentrations of progesterone from baseline varied with luteal phase from $9.95 \pm 1.33 \text{ ng ml}^{-1}$ for days 5–7 and $7.66 \pm 0.56 \text{ ng ml}^{-1}$ for days 8–12 to $4.35 \pm 0.56 \text{ ng ml}^{-1}$ for days 15–18 of the oestrous cycle ($n = 15$ for each group; mean $\pm$ SEM).

progesterone after stimulation was increased. In contrast, IGF-2 or insulin at the same dose as IGF-1 caused an acute release of progesterone that was limited to the time of the peptide infusion. This might suggest a different mode of action for IGF-1 and IGF-2 or insulin, that is possibly mediated by different types of receptor. Indeed, we found different types of receptor in the bovine luteal tissue. The presence and the typical binding characteristics of the type 1 IGF receptor (preferential binding of IGF-1, lower affinity for IGF-2 and binding of insulin only at much higher concentrations) was evident at all luteal stages. Additionally, type 2 IGF receptors were present throughout the luteal cycle. Although we found about twofold higher specific binding of IGF-2 compared with IGF-1, the limited significance of quantitative comparisons of different receptor

Table 2. Comparison of the areas under the curves of progesterone release (units in 4 h) after the beginning of the stimulation for 30 min with insulin-like growth factor (IGF)-1, IGF-2 or insulin in the luteal tissue of cows at three different luteal stages ($n = 5$; mean $\pm$ SEM)

	Days 5–7	Days 8–12	Days 15–18
IGF-1 (130 nmol l ⁻¹)	2889 $\pm$ 89 ^a	3123 $\pm$ 65 ^a	3944 $\pm$ 361 ^b
IGF-2 (130 nmol l ⁻¹)	2621 $\pm$ 77 ^{NS}	2719 $\pm$ 89	2957 $\pm$ 238
Insulin (1300 nmol l ⁻¹)	2816 $\pm$ 102 ^{NS}	3144 $\pm$ 163	2655 $\pm$ 191

^{a,b}Values with different superscripts in a row differ with $P < 0.05$.
^{NS}No significant difference between luteal stages.

Fig. 3. Effect of intraluteal application of (a) insulin-like growth factor (IGF)-1 (130 nmol l⁻¹), (b) IGF-2 (130 nmol l⁻¹) and (c) insulin (1300 nmol l⁻¹) on the release of oxytocin at different stages of the luteal phase *in vitro* (means $\pm$ SEM; $n = 5$ corpora lutea per group). ($\square$) values from pooled baselines (fractions 1–6), ($\blacksquare$) during peptide infusion (fraction 7–9) and ($\boxtimes$) after peptide infusion (fractions 10–15), respectively. Absolute oxytocin concentrations changed with the luteal stages from 71.2 ± 19.7 pg ml⁻¹ for days 5–7 and 48.7 ± 21.1 pg ml⁻¹ for days 8–12 to 1.4 ± 0.2 pg ml⁻¹ for days 15–18 of the oestrous cycle ($n = 15$ for each group; mean $\pm$ SEM). * $P < 0.05$ compared with the respective baseline values (100%).

types must be considered; even if there was no difference in the specific activities of IGF-1 and IGF-2 tracers as in our studies, there might be differences in the amount of tracer available for receptor binding.

Fig. 4. Competitive binding of (a) ^{125}I -labelled insulin-like growth factor (IGF)-1, (b) ^{125}I -labelled IGF-2, (c) ^{125}I -labelled insulin and ($\blacktriangle$) unlabelled IGF-1, ($\bullet$) IGF-2 and ($\blacksquare$) insulin to membrane preparations of bovine corpus luteum at days 8–12 of the oestrous cycle.

In the insulin binding experiments, we observed the characteristic two-site binding model with affinity constants that were in the same range as reported previously for insulin receptors (Hollenberg & Cuatrecasas, 1975). However, the relative potencies of unlabelled IGF-1 and IGF-2 in displacing the insulin tracer did not show the characteristic rank of order (insulin > IGF-1 > IGF-2) that has been described as typical for insulin receptors. Instead, all three unlabelled ligands showed similar potencies. It remains unclear whether the specific insulin binding in bovine luteal tissue is due to insulin receptors or whether type 1 IGF receptors account for this. Insulin receptors have been demonstrated in bovine granulosa cells (McArdle *et al.*, 1991) and this observation might support the presence of these receptors in luteal tissue. However, this issue cannot be resolved by binding studies; possibly the use of selective antibodies that are validated for bovine samples would be a useful way to resolve this question. Considering our receptor data and the stimulation experiments in the microdialysis perfusion system, the effects of IGF-1 on progesterone and oxytocin secretion are probably mediated via the specific type 1 receptor. An effect of IGF-1 mediated through insulin receptors should also be considered although the presence of insulin

Fig. 5. Representative Scatchard plots for the competitive binding of ($\blacktriangle$) ^{125}I -labelled insulin-like growth factor (IGF)-1 and unlabelled IGF-1, ($\bullet$) of ^{125}I -labelled IGF-2 and unlabelled IGF-2 and ($\square$) for ^{125}I -insulin and unlabelled insulin to membrane preparations of bovine corpus luteum at mid-luteal stage (days 8–12).

Fig. 6. Competitive binding of ^{125}I -labelled insulin-like growth factor (IGF)-2 and IGF-2 to membrane preparations from bovine corpus luteum at ($\diamond$) days 5–7, ($\blacksquare$) days 8–12 and ($\triangle$) days 15–18 of the oestrous cycle.

receptors in the bovine corpus luteum is not yet clear. Compared with IGF-1, the mode for IGF-2 action seems to be more complex. There are three possible interpretations of our results. First, IGF-2 may mediate its effects on luteal secretion through the type 2 receptor (binds exclusively IGF-2, negligible binding of both IGF-1 and insulin). Second, IGF-2 could act on luteal secretion through the type 1 receptor, although the effects seem to be different from those of IGF-1. Finally, IGF-2 may act via insulin receptors provided that this receptor type is expressed in the bovine corpus luteum. With regard to the stimulation in the microdialysis perfusion system, IGF-2 was as effective as insulin in this study. When the effects of IGF-2 on progesterone release are compared with those of insulin in the dose-response experiment, 13 nmol l^{-1} of both peptides showed very similar effects. This suggests that the action of IGF-2 could be mediated via insulin receptors, but it does not necessarily exclude the possibility that the type 2 receptor is dominantly concerned.

Insulin was also a very potent stimulator in our system. The data support the chronic effects of insulin in stimulating bovine luteal cells in long-term culture (McArdle & Holtorf, 1989). This group showed that the EC_{50} of insulin was only sevenfold higher than that for IGF-1, which is in agreement with our observations on the acute effects of these peptides. The existence of specific receptors for insulin in bovine corpora lutea is supported, since for type 1 IGF-receptor-mediated insulin responses a relatively higher insulin dose seems necessary to achieve the same reaction as that induced by IGF-1, which has a higher affinity for its receptor.

The effects of IGFs and insulin on luteal secretion changed with luteal stages. All three peptides were most effective in stimulating the relative peak heights of progesterone release in the late luteal stage of the oestrous cycle, whereas an effect on the total release of progesterone was limited to IGF-1 stimulation. The observation that corpora lutea of late luteal stage show the highest response is noteworthy since at this stage the basal progesterone release is lower than at other luteal phases. Factors such as IGF-1 are possibly important in the late luteal phase to ensure luteal function. With regard to the increasing intraluteal IGF-1 concentrations (Einspanier *et al.*, 1990) at this stage, it is possible that the role of IGF-1 is to maintain release of progesterone from corpora lutea that is sufficient for physiological function. However, the effects on oxytocin release showed a different profile: the IGFs tended to be less effective at the mid-luteal stage whereas insulin was most effective at early to mid-luteal stages. This observation supports the hypothesis that IGF-1 and insulin might act in different ways in luteal tissue.

The different effectiveness of IGF-1 in three luteal stages on luteal secretion cannot be attributed to differing type 1 receptor concentrations because there was no difference observed in either affinity or capacity of the IGF-1 receptor. Considering the endogenous luteal tissue IGF-1 concentrations (Einspanier *et al.*, 1990), which show a linear increase from days 1–5 until days 12–17, it is possible that the constant receptor population is saturated: taking 1% of total IGF-1 as freely available IGF-1 as estimated for plasma (Baxter & Martin, 1986) as a basis, additional exogenous free IGF-1 is potentially more effective in early corpora lutea compared with mid-luteal corpora lutea according to the law of mass action. However, nothing is known about the distribution of IGF-1 in luteal tissue, i.e. about the ratio of free and bound IGF-1. Additionally, a possible shift in the ratio of the different binding proteins during the oestrous cycle might affect the amount of IGF-1 available for receptor binding.

The changing effects of IGF-2 on progesterone and oxytocin secretion of tissue of corpora lutea during the oestrous cycle might partly be explained by a change in the population of type 2 receptor. The observation that IGF-2 at low concentrations increases the specific binding of ^{125}I -labelled IGF-2, as we found in corpora lutea at the late luteal phase, has been described previously in cell culture systems as well as in binding studies on isolated membranes (Marsh *et al.*, 1984; Barenton *et al.*, 1986). It might be attributed to a positive co-operative interaction, that is intrinsic to the receptor and to its oligomeric structure as concluded by Marsh *et al.* (1984). The possibility that binding proteins released by the cell might cause the described co-operativity (e.g. Barenton *et al.*, 1986) is unlikely because we used isolated membranes in our receptor studies. The positive co-operativity of IGF-2 binding to bovine corpora lutea membranes is also indicated by Scatchard

plots (De Lean & Rodbard, 1979); this feature could be seen not only in the late luteal stage but also in mid-luteal phase corpora lutea (Fig. 6), although specific binding was not increased at low IGF-2 concentrations in this case. However, this phenomenon is probably only one factor influencing the different action of IGF-2 in corpora lutea during the oestrous cycle. As discussed for IGF-1, the binding to related receptors, the presence of binding proteins as well as the endogenous concentration of the respective peptides are important but presently only partially known determinants for the effect of IGF-2.

In conclusion, the results of this study give further evidence that IGF-1 and related peptides may be important autocrine/paracrine regulators of the intraluteal function. The local expression of IGF-1 favours its role as the more important peptide. Further studies on the local production of IGF binding proteins, of IGF-2 and also of insulin receptors are needed to clarify the physiological role of the IGFs and insulin for the function of the corpus luteum.

A. Miyamoto was supported by the Deutscher Akademischer Austauschdienst (DAAD) and the Japan Society for the Promotion of Science (JSPS). We thank Ciba Geigy, Basel, Switzerland for the generous gift of rhIGF-1 and rhIGF-2; and Fresenius AG, St Wendel, Germany for the microcapillary membrane. This work was supported by the Deutsche Forschungsgemeinschaft (DFG).

References

- Adashi, E.Y., Resnick, C.E., Svoboda, M.E. & Van Wyk, J.J. (1984) A novel role for somatomedin-C in the cytodifferentiation of the ovarian granulosa cell. *Endocrinology* **115**, 1227–1229.
- Adashi, E.Y., Resnick, C.E., Svoboda, M.E. & Van Wyk, J.J. (1986) Follicle-stimulating hormone enhances somatomedin C binding to cultured rat granulosa cells. *Journal of Biological Chemistry* **261**, 3923–3926.
- Banskota, N.K., Carpentier, J.-L. & King, G.L. (1986) Processing and release of insulin and insulin-like growth factor I by macro- and microvascular endothelial cells. *Endocrinology* **119**, 1904–1913.
- Barenton, B., Patel, B.A., Guyda, H.J. & Posner, B.I. (1986) In *6th Annual Meeting of the Endocrine Society*, Abstract 211.
- Baxter, R.C. & Martin, J.L. (1986) Radioimmunoassay of growth-hormone dependent insulin-like growth factor binding protein in human plasma. *Journal of Clinical Investigation* **78**, 1504–1512.
- Chou, S.Y., Kostyo, J.L. & Adamofio, N.A. (1990) Growth hormone inhibits activation of phosphatidylinositol phospholipase C by insulin in ob/ob mouse adipose tissue. *Endocrinology* **126**, 62–66.
- Davoren, J.B. & Hsueh, J.W. (1986) Growth hormone increases ovarian levels of immunoreactive somatomedin C/insulin-like growth factor I in vitro. *Endocrinology* **118**, 888–890.
- Davoren, J.B., Kasson, B.G., Li, C.H. (1985) Somatomedin C augments FSH-induced differentiation of cultured rat granulosa cells. *American Journal of Physiology* **249**, 26–33.
- Davoren, J.B., Kasson, B.G., Li, C.H. & Hsueh, A.J.W. (1986) Specific insulin-like growth factor (IGF) I- and II-binding sites on rat granulosa cells: relation to IGF action. *Endocrinology* **119**, 2155–2162.
- De Lean, A. & Rodbard, D. (1979) Kinetics of cooperative binding. In *The Receptors*, Vol. 1, pp. 143–192. Ed. R. D. O'Brien. Plenum Press, New York.
- Einspanier, R., Miyamoto, A., Schams, D., Müller, M. & Brem, G. (1990) Tissue concentration, mRNA expression and stimulation of IGF-I in luteal tissue during the oestrous cycle and pregnancy of cows. *Journal of Reproduction and Fertility* **90**, 439–445.
- Hammond, J.M., Baranano, J.L.S., Skaleris, D., Knight, A.B., Romanus, J.A. & Rechler, M.M. (1985) Production of insulin-like growth factors by ovarian granulosa cells. *Endocrinology* **117**, 2553–2555.
- Hoffmann, B., Kyrein, H.J. & Ender, M.L. (1973) An efficient procedure for the determination of progesterone by radioimmunoassay applied to bovine peripheral plasma. *Hormone Research* **4**, 302–310.
- Hollenberg, M.D. & Cuatrecasas, P. (1975) Methods for the biochemical identification of insulin receptors. In *Methods in Receptor Research*, Vol. 9, pp. 429–478. Ed. M. Blecher. M. Dekker, Inc., New York and Basel.
- Jarry, H., Dietrich, M., Düker, E. & Wuttke, W. (1987) Effects of systemic and local administration of etomidate on adrenocortical steroidogenesis in male rats. *Acta Endocrinologica* **114**, 402–409.
- McArdle, C.A. & Holtorf, A.P. (1989) Oxytocin and progesterone release from bovine corpus luteal cells in culture: effects of insulin-like growth factor-I, insulin and prostaglandins. *Endocrinology* **124**, 1278–1286.
- McArdle, C.A., Kohl, C., Rieger, K., Gröner, I. & Wehrenberg, U. (1991) Effects of gonadotropins, insulin and insulin-like growth factor I on ovarian oxytocin and progesterone production. *Molecular and Cellular Endocrinology* **78**, 211–220.
- McPherson, G.A. (1985) Analysis of radioligand binding experiments. *Journal of Pharmacological Methods* **14**, 213–228.
- Marsh, J.W., Westley, J. & Steiner, D.F. (1984) Insulin-receptor interactions. *Journal of Biological Chemistry* **259**, 6641–6649.

- Miyamoto, A. & Schams, D. (1991) Oxytocin stimulates progesterone release from microdialyzed bovine corpus luteum in vitro. *Biology of Reproduction* **44**, 1163–1170.
- Munson, P.J. & Rodbard, D. (1980) LIGAND: A versatile computerized approach for characterization of ligand-binding systems. *Analytical Biochemistry* **107**, 220–239.
- Prakash, B.S., Meyer, H.H.D., Schallenberger, E. & Van de Wiel, D.F.M. (1987) Development of a sensitive enzymeimmunoassay (EIA) for progesterone determination in unextracted bovine plasma using the second antibody technique. *Journal of Steroid Biochemistry* **28**, 623–627.
- Rechler, M.M. (1989) Molecular insights into insulin-like growth factor biology. *Journal of Animal Science* **66** (Supplement 3), 76–83.
- Rinderknecht, E. & Humbel, R.E. (1978) The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. *Journal of Biological Chemistry* **253**, 2769–2776.
- Savion, N., Lui, G., Laherty, R. & Gospodarowicz, D. (1981) Factors controlling proliferation and progesterone production by bovine granulosa cells in serum-free medium. *Endocrinology* **109**, 409–420.
- Scatchard, G. (1949) The attractions of proteins for small molecules and ions. *Annals of New York Academy of Sciences* **51**, 660–675.
- Schams, D. (1983) Oxytocin determination by radioimmunoassay. *Acta Endocrinologica* **103**, 180–183.
- Schams, D., Koll, R. & Li, C.H. (1987) Insulin-like growth factor-I stimulates oxytocin and progesterone production by bovine granulosa cells in culture. *Journal of Endocrinology* **116**, 97–100.
- Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. & Klenk, D.C. (1985) Measurement of protein using bicinchoninic acid. *Analytical Biochemistry* **150**, 76–85.
- Takasu, N., Komiya, I., Nagasawa, Y., Asawa, T., Shimizu, Y. & Yamada, T. (1990) Cytoplasmic pH in the action of insulin-like growth factor-I in cultured porcine thyroid cells. *Journal of Endocrinology* **127**, 305–309.
- Thorell, J.I. & Johansson, B.G. (1971) Enzymatic iodination of polypeptides with ¹²⁵I to high specific activity. *Biochemica et Biophysica Acta* **25**, 363–369.
- Veldhuis, J.D., Furlanetto, R.W., Juchter, D., Garmey, J. & Veldhuis, P. (1985) Trophic actions of human somatomedin C/insulin-like growth factor I on ovarian cells: in vitro studies with swine granulosa cells. *Endocrinology* **116**, 1235–1242.

Received 9 July 1991