Relationship between pituitary GnRH-binding sites and pituitary release of gonadotrophins in post-partum beef COWS

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Summary. Thirty primiparous suckling beef cows were slaughtered on Day 7, 14, 28, 42 or 56 after parturition. Some had resumed oestrous cyclicity by the time they were slaughtered on Days 42 and 56. Amongst acyclic cows between Days 7 and 42, pituitary LH concentrations and basal and GnRH-induced release of LH from pituitary explants doubled. Pituitary FSH concentration and basal release in FSH increased only by 15–20%, while GnRH-induced release of FSH *in vitro* was unchanged. During post-partum anoestrus, overall mean concentrations of serum FSH did not change, whereas overall mean concentrations and pulse amplitudes of serum LH increased. Numbers and affinity constants of GnRH-binding sites in pituitary glands remained constant during the post-partum period studied. We conclude that, under these experimental conditions, numbers and affinity constants of GnRH-binding sites in the pituitary gland of post-partum beef cows do not limit the ability of the anterior pituitary gland to release gonadotrophins.

Introduction

After parturition, beef cattle go through a period of anoestrus before resumption of oestrous cyclicity. Insufficient secretion of LH from the anterior pituitary gland is one reason for anoestrus. This view is based upon the following observations. After calving, pituitary LH content (Labhsetwar, Collins, Tyler & Casida, 1964; Saiduddin, Riesen, Tyler & Casida, 1968; Cermak, Braden, Manns, Niswender & Nett, 1983), GnRH-induced release of LH (Kesler, Garverick, Youngquist, Elmore & Bierschwal, 1977; Webb, Lamming, Haynes, Hafs & Manns, 1977; Fernandes, Thatcher, Wilcox & Call, 1978) and mean concentrations of LH in serum (Peters, Lamming & Fisher, 1981; Williams *et al.*, 1982) were lower than those observed just before the first post-partum oestrus. Similar changes in these indices of LH secretion occur in sheep (Jenkin, Heap & Symons, 1977). Gradual restoration of GnRH-induced release of LH in women during the puerperium has also been reported (Jeppsson, Rannevik & Kullander, 1974; LeMaire, Shapiro, Riggall & Yang, 1974).

Binding of GnRH to specific sites in the plasma membrane of gonadotrophs is the first step in stimulation of LH secretion. Any changes in either the number or affinity constant of the GnRH-binding sites may alter the sensitivity of gonadotrophs to GnRH. Therefore, reduced LH

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secretion during the post-partum period in cattle may be due to reduced numbers and/or affinity of GnRH-binding sites. Clayton, Solano, Garcia-Vela, Dufau & Catt (1980) and Clayton & Catt (1981) have reported that lactating rats have about half the number of pituitary GnRH-binding sites that are found in rats in metoestrus; affinity constants did not change. In the experiment described herein, we evaluated whether the ability of the pituitary gland of post-partum beef cows to release gonadotrophins in response to GnRH was related to the number and affinity constant of GnRH-binding sites in the same pituitary gland.

Materials and Methods

Animals

Primiparous crossbred (primarily Hereford × Angus) cows were obtained from the Upjohn Company (Kalamazoo, MI, U.S.A.). They were about 2 years old when artificially inseminated between 22 September and 16 October after synchronization of oestrus with prostaglandin F-2 α (Upjohn, Kalamazoo, MI, U.S.A.). These cows averaged 479 ± 13 kg body weight and had a score of 7 out of a possible 10 for body condition (fat) just before parturition. Cattle were fed to meet requirements established by the National Research Council (1976). Feeds consisted of corn silage supplemented with vitamins and minerals. All cows were 'halter-broken' and accustomed to handling before parturition. Parturition occurred between 1 and 24 July. Each cow suckled a single calf until slaughter.

The cows were assigned randomly to be slaughtered on Days 7, 14, 28, 42 or 56 after parturition. Housing was in groups of 5–6 cows per pen.

Blood samples

A pen was modified for collection of blood samples from an adjacent room via a remote cannula fitted into a jugular vein of each cow. Cows and calves were acclimatized to this pen 4 days before collection of blood. On the day before slaughter, cows were bled at 10-min intervals between 08:00 and 14:00 h. During the sampling period, cows were loosely restrained with halters and calves had free access to dams. Blood was stored for 2 h at room temperature, then at 4°C for an additional 24 h before centrifugation to obtain serum. Serum was stored at –20°C until concentrations of LH and FSH were measured.

Beginning on Day 21 after parturition, blood was also collected by venepuncture every 3 days until time of slaughter. These samples were assayed for progesterone. Serum concentrations of progesterone greater than 1 ng/ml and the presence of newly formed corpora lutea at slaughter were used to estimate when these cows resumed oestrous cycles.

Anterior pituitary glands

Pituitary glands were collected and packed in ice within 15 min after cows were killed. Anterior pituitary glands were isolated and bisected. Pituitary halves from each animal were weighed and then randomly assigned either to be stored in liquid nitrogen until quantification of GnRH-binding sites or to be incubated *in vitro* to determine the ability of the gland to release gonadotrophins.

Quantification of GnRH-binding sites in anterior pituitary glands. A hemi-pituitary gland from each cow was homogenized in 15 ml Tris-HCl buffer (pH 7.7, 10 mM-Tris with 1 mM-dithiothreitol) using an Omni-mixer (50 ml container; Sorvall, Norwalk, CT, U.S.A.) at maximum speed for 2 min. The resulting slurry was homogenized further with a hand-driven tissue grinder (glass to glass, Pyrex No. 7726, 0.15 mm clearance, 10 strokes): 200 μ l of this homogenate were stored at –70°C until assayed for LH and FSH. A crude membrane fraction was prepared from the remaining homogenate as described previously (Leung, Padmanabhan, Convey, Short & Staigmiller,

1984). Binding assays for GnRH were carried out for individual hemi-pituitaries, and Scatchard analyses were subsequently performed. Numbers of binding sites are expressed as fmol/mg membrane protein. Protein was assayed as described by Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as standard.

GnRH analogue, D-Ala⁶-des-Gly¹⁰-GnRH ethylamide (GnRH-A; Peninsula Laboratories, San Carlos, CA, U.S.A.) was iodinated by a lactoperoxidase–glucose oxidase method (Clayton *et al.*, 1979). Specific activity was calculated as described previously (Leung *et al.*, 1984) and ranged from 1152 to 2017 µCi/µg. The maximal percentage of specific binding of ¹²⁵I-labelled GnRH-A to an excess of membrane protein varied with iodinations and ranged from 28 to 43%. Total radioactivity added to each assay tube was corrected for variation in maximal specific binding before Scatchard analysis. The assay was carried out as previously reported (Leung *et al.*, 1984) and radioactivity was measured in a gamma spectrometer with a counting efficiency of 85%.

Fresh and frozen anterior pituitary glands. Before assay we planned to hold all pituitary glands in liquid nitrogen until the end of the experiment when all the heifers were killed. But we were concerned that numbers and/or affinity of GnRH-binding sites might change with freezing. Therefore, we performed a preliminary experiment to test this question. Anterior pituitary glands were obtained from cows killed at a local abattoir, bisected, and the halves were randomly assigned to be used fresh or after freezing in liquid nitrogen for 2 h followed by thawing over a 15-min period. Binding assays were run on each pituitary half. Neither the affinity constants (0.96 ± 0.12 and $0.98 \pm 0.12 \times 10^{10}$ M) nor numbers (94.4 ± 22.8 and 99.1 ± 22.5 fmol/mg protein) of GnRH-binding sites in the crude membrane preparation from frozen pituitary halves were different ($P < 0.25$) from those prepared from fresh halves. Pituitary halves were therefore stored in liquid nitrogen until time of assay (34–40 weeks). GnRH-binding sites in all pituitary glands were quantitated within a single assay. Intra-assay coefficient of variation was 5.9%.

Ability of the anterior pituitary gland to release gonadotrophins. To determine changes in ability of anterior pituitary glands to release LH and FSH, one half of the fresh anterior pituitary gland from each animal was sliced and diced into 1–2 mm³ explants. These pituitary explants (20–25 µg) were incubated in 4 ml culture medium contained in a 25 ml Erlenmeyer flask under an atmosphere of 95% O₂ and 5% CO₂ at 37°C. Culture medium consisted of Hank's minimum essential medium and Medium 199 in 1:1 (v/v) ratio supplemented with 5 mM-L-glutamine (Gibco, Grand Island, NY, U.S.A.). Explants were first washed with 4 ml culture medium at 15-min intervals for 1 h and then incubated in the absence of any treatment for 2 h to establish basal release of gonadotrophins into medium. At the end of 2 h, the medium was changed and explants were challenged with 4 ng GnRH (Beckman, Palo Alto, CA, U.S.A.) per flask for an additional 2 h. Again, medium was collected after the challenge. All samples of medium were assayed for LH and FSH.

Radioimmunoassays

Concentrations of LH in all serum and medium samples were determined in 4 assays using a double-antibody procedure previously validated in this laboratory (Convey, Beal, Seguin, Tannen & Lin, 1976). Standard LH was NIH-LH-B8. Inter- and intra-assay coefficients of variation from 4 assays were 12.1 and 11.6%, respectively. Sensitivity of the LH assay was 0.125 ng/tube.

Serum and medium FSH were quantified by radioimmunoassay using USDA-FSH-B1 as standard. Rabbit anti-bovine FSH serum (B-5; 1:75 000 dilution) and highly purified bovine FSH were supplied by Dr K. W. Cheng, University of Manitoba, Winnipeg, Manitoba, Canada. The assay was performed as previously described (Carruthers, Convey, Kesner, Hafs & Cheng, 1980).

Hormone specificity of binding of ¹²⁵I-labelled bovine FSH to the FSH antiserum was determined in the presence of various amounts of bovine TSH (Dr J. G. Pierce, University of California at Los Angeles, U.S.A.), bovine LH (R-107, Dr L. E. Reichert, Albany Medical College, NY, U.S.A.), bovine FSH, NIH-GH-B18, NIH-prolactin-B5 and USDA-FSH-B1 (Fig. 1). Cross-reaction was calculated as follows:

Fig. 1. Cross-reaction of anti-bovine FSH serum with various pituitary hormone preparations. Description of hormone preparations is in 'Materials and Methods'.

$$\frac{\text{amount of USDA-FSH-B1 required to reduce binding of } ^{125}\text{I-labelled FSH to 50\%}}{\text{amount of other pituitary hormone required to reduce binding of } ^{125}\text{I-labelled FSH to 50\%}} \times 100\%$$

Cross-reaction of the antiserum with bovine prolactin and GH was <0.5%, while cross-reactions with bovine TSH and LH were about 2.6%. Highly purified bovine FSH was 29 times more potent than USDA-FSH-B1 in displacing bound ^{125}I -labelled FSH from the antiserum.

Displacement of ^{125}I -labelled FSH by increasing volumes of cow serum (Day 3 of oestrous cycle), ovariectomized heifer serum and media from cultures of pituitary explants (two pools) was parallel to the standard curves (Fig. 2). Known quantities of FSH (NIH-FSH-B1) were added to cow serum, ovariectomized heifer serum and culture medium: recoveries were 125, 132 and 93%, respectively.

Sensitivity of the FSH assay was 2.5 ng/tube. Inter- and intra-assay coefficients of variation from 3 assays were 10.8 and 8.1% respectively.

Statistical analysis

Acyclic vs cyclic cows. At slaughter, 10 of the 30 cows had corpora lutea and concentrations of serum progesterone > 1 ng/ml, indicating resumption of oestrous cyclicity. Therefore, data from the cows were arranged into the following six groups: those that were acyclic on post-partum Days 7 (N = 6), 14 (6), 28 (5) or 42 (3) and those that were cyclic on post-partum Days 42 (5) or 56 (5). Since beef cows resumed oestrous cyclicity on different post-partum days and were slaughtered on fixed post-partum days, means of variables obtained on Days 42 and 56 from cyclic cows represented different days of the oestrous cycle.

Evaluation of serum hormones. The following criteria were used:

pulse = one or more consecutive values that exceeded a preceding value by 3 standard deviations (s.d.) established from control serum values run in the same assay (3 s.d. = 0.3 ng/ml for LH and 7.5 ng/ml for FSH);

nadir = lowest point(s) between two defined and adjacent pulses;

baseline for animals with pulsatile release = mean of all samples that were equal to nadir or within the range of nadir $\pm$ assay sensitivity;

baseline for animals with no pulsatile release = mean of all serum samples;

amplitude = the difference between maximal value reached during a pulse and the nadir preceding the pulse.

Analysis of data

All data reported were tested for heterogeneity of variance amongst groups using Bartlett's test (Gill, 1978). Heterogeneity was found in variances of baseline concentrations and overall mean concentrations of serum LH. Therefore, data of these two variables were subjected to natural logarithmic transformation before statistical analysis. Data are presented untransformed. All data were analysed by one-way analysis of variance. Specific comparisons of means were conducted using Bonferroni's *t* test (Gill, 1978).

Fig. 2. FSH standard curve and inhibition curves by bovine sera and media from bovine pituitary explant cultures. Description of materials is in 'Materials and Methods'.

Results

Cows and calves

Thirteen bull calves and 17 heifer calves were born. Sex and birth weight of the calves and gestation lengths did not differ amongst groups ($P > 0.25$). Calves had a mean ($\pm$ s.e.m.) birth weight of 26.4 ± 0.6 kg, and gestation lengths averaged 277.8 ± 0.6 days. No major calving difficulty was encountered.

In-vitro release of gonadotrophins

Basal release of LH and FSH. Among acyclic cows between Days 7 and 42 after parturition, basal release of LH into the medium increased linearly ($P < 0.005$) from 9.1 to 20.2 ng/ml medium/mg pituitary gland, and basal release of FSH increased linearly ($P < 0.025$) from 136 to 164 ng/ml medium/mg pituitary gland (Fig. 3). By Days 28 to 42 after parturition, basal releases of LH and FSH from acyclic cows were comparable ($P > 0.20$) to those of cyclic cows on Days 42 and 56 after parturition.

Fig. 3. Concentration of basal and GnRH-induced release of LH and FSH from pituitary explants of post-partum beef cows. Values are means $\pm$ s.e.m.

Fig. 4. Concentrations of LH and FSH in anterior pituitary glands of post-partum beef cows. Values are means $\pm$ s.e.m.

GnRH-induced release of LH and FSH. The profile of GnRH-induced release of LH from pituitary explants paralleled basal release of LH (Fig. 3). There was a linear increase ($P < 0.05$) in GnRH-induced release of LH into medium from 17.1 to 34.8 ng/ml medium/mg pituitary gland between Days 7 and 42 after parturition. GnRH-induced release of LH in acyclic cows on Days 28 to 42 after parturition was similar ($P > 0.20$) to that of cyclic cows on Days 42 and 56 after parturition. GnRH-induced release of FSH into medium, however, did not vary ($P > 0.20$) between Days 7 and 56 after parturition within acyclic cows or between acyclic and cyclic cows (Fig. 3).

Concentrations of LH or FSH in medium in response to GnRH did not change ($P > 0.20$) between Days 7 and 42 after parturition when expressed as the difference between basal and GnRH-induced release of LH or FSH from pituitary explants.

Pituitary concentrations of LH and FSH

Within acyclic cows, pituitary LH concentrations ($\mu\text{g/mg}$ pituitary tissue) increased from 0.43 to 0.76 and pituitary FSH concentrations from 0.85 to 0.99 between Days 7 and 42 after parturition (Fig. 4). Both increases were linear ($P < 0.05$). By Days 28 to 42 after parturition, pituitary concentrations of LH of acyclic cows were similar ($P > 0.20$) to those of cyclic cows on Days 42 and 56 after parturition. Pituitary gland concentrations of FSH did not vary ($P > 0.20$) between cyclic and acyclic cows.

There were positive correlations ($P < 0.001$) between LH concentrations in pituitary glands and basal ($r = 0.76$) and GnRH-induced ($r = 0.75$) releases of LH from pituitary explants in culture. A positive correlation was also detected ($r = 0.53$; $P < 0.005$) between FSH concentration in the pituitary gland and basal release of FSH from the explants.

GnRH-binding sites

Affinity constants and numbers of GnRH-binding sites in the pituitary glands did not change with time after parturition or between acyclic and cyclic cows ($P > 0.25$, Table 1).

Table 1. Mean ($\pm$ s.e.m.) affinity constants and numbers of GnRH-binding sites in anterior pituitary glands of post-partum beef cows

Status	Days post partum	No. of cows	Affinity constants ($\times 10^{10} \text{ M}^{-1}$)	No. of binding sites (fmol/mg protein)
Acyclic	7	6	0.79 ± 0.09	98.3 ± 13.8
	14	6	0.73 ± 0.05	119.9 ± 15.7
	28	5	0.67 ± 0.06	129.0 ± 18.6
	42	3	0.81 ± 0.04	81.8 ± 12.3
Cyclic	42	5	0.71 ± 0.03	136.6 ± 25.8
	56	5	0.81 ± 0.07	94.8 ± 11.4
			0.75 ± 0.02	110.7 ± 7.4

Indices of serum gonadotrophins

LH. Indices of serum LH are presented in Fig. 5. Frequency of pulses and mean baseline concentrations of LH did not change with time after parturition, or when oestrous cyclicity resumed ($P > 0.20$). Although overall mean concentrations of LH tended to be lower on Day 7 than on Days 14 to 42, these differences only approached significance ($P > 0.10$). The mean amplitude of LH pulses observed on Days 14, 28 and 42 after parturition was greater ($P < 0.05$) than that on Day 7 (2.4 ± 0.2 vs 1.3 ± 0.4 ng/ml, respectively). The average amplitude of pulsatile LH release between

Fig. 5. Indices of serum LH in post-partum beef cows. Values are means $\pm$ s.e.m. for the no. of cows in parentheses.

Days 7 and 42 after parturition amongst acyclic cows was higher ($P < 0.05$) than that of cyclic cows (2.1 ± 0.2 vs 1.3 ± 0.3 ng/ml respectively).

FSH. Frequencies and amplitudes of the FSH pulses, mean baseline concentrations and overall mean concentrations of serum FSH did not change ($P > 0.20$) among all days studied.

Discussion

Under the in-vitro conditions in the present study, the test of pituitary response to a presumed maximal dose of GnRH revealed that concentrations of medium LH after GnRH were about 1.9 times that before GnRH for all times studied. Therefore, in this study, an increase in release of LH with time from parturition was probably due to increased ability of the pituitary gland to release LH. This increase in the ability of the pituitary gland to release LH was not accompanied by alteration in either the affinity constant or number of GnRH-binding sites in the pituitary gland. There is therefore no direct relationship between numbers and affinity constants of GnRH-binding sites in the pituitary gland of post-partum beef cows and the ability of the gland to release gonadotrophins. This observation is similar to that deduced by Leung *et al.* (1984) for cattle and by Ferland *et al.* (1981) for rats during pro-oestrus of their respective cycles. However, the number of GnRH-binding sites was greater in the present study than we have previously reported (Leung *et al.*, 1984) and we consider that variation amongst animals may account for the difference.

Evidence suggests that the number of GnRH-binding sites is in excess, i.e. occupancy of only a fraction of GnRH-binding sites is required for maximal LH release. Naor, Clayton & Catt (1980) showed that occupancy of 20% of GnRH-binding sites elicited about 80% of the maximal LH release from cultured rat pituitary cells. Therefore, even with the variation in the number of GnRH-binding sites observed in the present study, there may be sufficient numbers of binding sites to mediate maximal release of LH.

In other studies (Savoy-Moore, Schwartz, Duncan & Marshall, 1980; Clayton *et al.*, 1980), GnRH-binding sites were measured in a crude fraction of membranes of the anterior pituitary gland. This crude membrane preparation consists of plasma membrane as well as other intracellular membranes. Consequently, other GnRH-binding sites not associated with plasma membrane may also be measured in their assays; for example, GnRH-binding to secretory granules (Sternberger & Petralli, 1975; Morel, Barry & Dubois, 1980) and nuclei (Millar, Rosen, Badminton, Pasqualini & Kerdelhue, 1983) has been reported. However, this concern does not apply to the present study. Most of the secretory granules and nuclei were removed from the final preparation of membrane by differential centrifugation. Even with this modification, however, no change in either the number or affinity constant of GnRH-binding sites in these preparations was detected. Therefore, our data provide convincing evidence for the discordant relationship between the ability of the pituitary gland to release gonadotrophins and number and affinity constant of GnRH-binding sites in the same pituitary gland.

Moss, Adams, Niswender & Nett (1980) reported that a constant percentage of total LH was released from dispersed pituitary cells of post-partum ewes after stimulation with a maximal dose of GnRH. These authors suggested that only a certain percentage of LH in the anterior pituitary gland was readily releasable. If this hypothesis is correct, as the concentrations of pituitary LH increase during post-partum anoestrus, we would expect increased amounts of LH to be available for release. Indeed, this phenomenon may explain the positive correlations in the present study between concentrations of LH in the pituitary gland and either basal or GnRH-induced release of LH from pituitary explants. Furthermore, the bulk of the evidence in the present study and that of Peters *et al.* (1981) and Williams *et al.* (1982) suggest that there is an increase in overall mean concentrations of LH in serum shortly after parturition. In addition, we observed an increase in amplitude of LH pulses. These increases in storage and release of LH may be prerequisite for the re-establishment of the first post-partum preovulatory surges of gonadotrophins.

The 20% increase in basal release of FSH from pituitary explants during post-partum anoestrus coincided with a 15% increase in concentrations of pituitary FSH during the same period. Although these increases in basal release of FSH from explants and concentrations of pituitary FSH were significant statistically, the physiological importance of these quantitatively small increases remains to be determined. In the present study, these increases were not reflected in changes in the concentrations of serum FSH.

We conclude that, under these experimental conditions, numbers and affinity constants of GnRH-binding sites in the pituitary gland of post-partum beef cows do not limit the ability of the gland to release gonadotrophins.

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