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Age-related changes in the secretion of LH *in vivo* and *in vitro* in infantile and prepubertal Holstein bull calves

J. M. McAndrews*, J. L. Peters and D. R. Deaver†

Department of Dairy and Animal Science, The Pennsylvania State University, University Park, PA 16802, USA

The objectives of this study were (i) to determine whether age-related changes in the secretion of LH are associated with alterations in secretory activity or numbers of gonadotrophs, and (ii) to determine whether gonadotrophs obtained from the pars distalis and pars tuberalis undergo similar age-related changes in function. Blood samples were collected from Holstein bull calves every 15 min for 12 h at <1, 12, and 42 weeks of age ($n = 5$ per age group) to characterize the secretion of LH. Calves were killed 3–5 days later. The pars distalis and pars tuberalis were enzymatically dispersed into suspensions of single cells. Cells from the pars distalis were (i) extracted with 0.01 mol $NaHCO_3$ l^{-1}, (ii) fixed for immunocytochemical analysis, and (iii) cultured in six-well plastic plates at a density of 500 000 cells per well in media containing 2.5% homologous calf serum for 18 and 72 h. Cells from the pars tuberalis were cultured as for pars distalis cells. As expected, LH pulse frequency increased ($P < 0.01$) between one and 12 weeks of age and then declined. The percentage of cells from the pars distalis containing immunoreactive LH averaged 8.4%, and did not change with age. The mass of the pars distalis and the total number of cells recovered increased with age ($P < 0.05$); consequently, the number of gonadotrophs recovered also increased. The initial content of LH of pars distalis cells changed with age and was greatest at 12 weeks. At <1 and 12 weeks of age, the difference in initial content was reflected in the amount of LH released *in vitro* after both 18 and 72 h in culture. In addition, at these ages the total amount of LH present (media plus cell extract) at the end of the culture period was similar to the initial cell content. In contrast, the total LH content of cells obtained from the pars distalis of calves at 42 weeks of age increased by approximately 3.5 times during the first 18 h of culture. The total content of LH from pars tuberalis cells after 18 h in culture increased slightly with age but this increase was not significant. In summary, age-related differences in the numbers and secretory activity of gonadotrophs located within the pars distalis contribute to age-related increases in concentrations of LH in plasma. Changes in the synthesis and release of LH from pars distalis and pars tuberalis cells appear to differ in culture.

Introduction

Secretion of LH undergoes complex changes between birth and puberty in Holstein bull calves. As described by Amann (1983), the sexual development of the bull can be divided into three stages prior to attainment of puberty: infantile, juvenile and prepubertal. Patterns of LH and steroid secretion differ in each of these stages. The infantile stage occurs from birth to approximately 10 weeks of age, and during this time discharges of LH occur infrequently (McCarthy *et al.*, 1979; Amann and Walker, 1983), and low circulating concentrations of testosterone are observed. The juvenile period begins at approximately 10–12 weeks of age in Holstein bull calves when there is a marked increase in the frequency and amplitude of discharges of LH (Amann and Walker, 1983; Deaver and Peters, 1988). These calves enter a prepubertal period at approximately 24 weeks of age when pulsatile secretion of LH decreases, presumably owing to the establishment of testosterone-mediated negative feedback (Deaver and Peters, 1988). They generally attain puberty at approximately 42 weeks of age (Amann, 1983).

While changes in plasma concentrations of LH and hypothalamic discharge of GnRH have been characterized, there is a paucity of data concerning pituitary content of LH and numbers of gonadotrophs during discrete stages of development. A major objective of this study was to correlate age-related changes in concentrations of LH in plasma with the secretion of LH *in vitro*. Specifically, it was determined

*Present address: Department of Neurobiology and Physiology, Northwestern University, 2153 Sheridan Road, Evanston, IL 60208, USA
†Correspondence and reprint requests.
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whether age-related changes in concentrations of LH in plasma were associated with increased numbers or secretory activity of gonadotrophs. A second objective was to determine whether gonadotrophs obtained from the pars distalis and pars tuberalis underwent similar age-related changes in function.

Materials and Methods

General methods

All Holstein bull calves used for this study were 3–5 days old and raised in individual pens at the Pennsylvania State University Dairy Breeding Research Center (University Park, PA), as previously described (Curtis and Amann, 1981), except that after 30 weeks of age the diet contained 16% crude protein. Animals were killed by exsanguination at the Penn State University Meats Laboratory at ages <1, 12 and 42 weeks ($n = 5$ per group). Three to five days before slaughter, blood samples were collected via an indwelling jugular catheter every 15 min beginning at 06:00 h and continuing until 18:00 h. Blood was collected in heparinized tubes and held on ice until centrifuged at 1200 ***g*** for 20 min. Plasma was divided into aliquots and kept at − 20°C until assayed for LH.

Pituitary collection, dispersion and culture

The pituitary gland was removed using an aseptic technique, and placed into sterile Eagle's modified minimum essential medium (SMEM, Gibco Laboratories, Grand Island, NY), supplemented with 0.1% BSA (Fraction V, Sigma Chemical Company, St Louis, MO). Pituitary glands were transported to the laboratory within 15 min of slaughter.

The pars distalis and pars tuberalis were weighed, minced into 3 mm × 3 mm pieces and transferred to separate spinner flasks. Approximately 50 ml of SMEM, 0.3% BSA, 0.3% trypsin (1:250, Difco, Detroit, MI) at a pH of 7.8 was added to the flask. Deoxyribonuclease (DNAse, 100 µl, 2 mg ml^{-1}, Sigma) was added to the flasks to prevent aggregation of liberated cells. The flasks were continuously agitated and incubated at 37°C for 45 min. After incubation the suspensions were transferred to 50 ml sterile plastic centrifuge tubes (Elkay Products, Shrewsbury, MA) and centrifuged at 200 ***g*** for 10 min. The supernatant was discarded and soybean trypsin inhibitor (100 µl, 0.1 mg l^{-1}, Sigma Chemical Co) was added to the pellets to inactivate any remaining trypsin. Approximately 10 ml of SMEM with 0.1% BSA was added and tissue pieces were triturated. Intact pieces of tissue were returned to the spinner flask and the dispersion procedure was repeated until all tissue was completely dissociated. In most cases 3–4 dispersion steps were needed for the complete dissociation of the pars distalis, while two steps were required for the pars tuberalis. Cell suspensions from each dispersion step were combined and centrifuged at 200 ***g*** for 10 min. The supernatant was discarded, and cells were resuspended in fresh SMEM with 0.1% BSA. An aliquot of the cells from the suspension was counted using a haemocytometer, the cell yield determined, and the suspension was adjusted to a concentration of 1×10^6 cells ml^{-1}.

Aliquots of cells from the pars distalis were extracted with 0.01 mol $NaHCO_3$ l^{-1} to determine the initial content of LH, and an aliquot was fixed in formaldehyde-buffered PBS for immunocytochemical analysis. Since the number of cells from the pars tuberalis was limited, these cells were subjected to the culture protocol only.

Cells from the pars distalis and pars tuberalis were cultured for 18 and 72 h in six-well plastic culture plates (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) at a density of 500 000 cells per well in 3.5 ml culture media. The culture media used was the α-modification of Eagle's minimum essential medium (α-MEM, Flow Laboratories, McLean, VA) supplemented with 2.5% homologous calf serum. Cells were incubated at 37°C in a humidified atmosphere of 95% air:5% CO_2. At 18 and 72 h after the initiation of the culture, media was removed and centrifuged at 4°C, at 1200 ***g*** for 30 min. Cultured cells were extracted with 1.5 ml of 0.01 mol $NaHCO_3$ l^{-1} to determine cellular content of LH. Plates were incubated at 4°C and 24 h later suspensions were centrifuged at 4°C, 1200 ***g***, for 30 min. All supernatant solutions of cell extracts and media were divided into aliquots and frozen at − 80°C until required for radioimmunoassay for LH.

Fixation, staining and flow cytometry of cells

The flow cytometric immunofluoresence staining of pars distalis cells was performed as described by Hatfield and Hymer (1986). An aliquot of dispersed pars distalis cells was fixed with PBS-buffered 4% (w/v) paraformaldehyde for 30 min. Cells were then washed four times with PBS–azide (0.01% w/v), 0.1% BSA, Triton X-100 (0.025% w/v), each wash step lasting 20 min. After washing, the suspension of cells was adjusted to a final concentration of 4×10^6 cells ml^{-1} in the PBS–azide/BSA/Triton X-100 buffer. Cells were stained for bovine LH by incubating 4×10^5 cells with 500 µl of a 1:2000 dilution of the primary antisera (rabbit anti-bovine LH-USDA-309-684-P; D. J. Bolt and USDA Hormone Distribution Program) and incubating for 12–16 h at 22°C. Nonspecific binding tubes were incubated with 500 µl of a 1:2000 dilution of normal rabbit sera. After incubation with the primary antisera (or normal rabbit sera for nonspecific binding tubes), cells were rinsed and subsequently incubated with 500 µl of a 1:400 dilution of fluorescein-isothiocyanate conjugated goat anti-rabbit IgG (Organon Teknika, West Chester, PA) for 1 h at 22°C. Cells were then rinsed and incubated with 25 µl RNAse (40 µg ml^{-1}, Serva Fine Biochemicals, Inc., Westbury, NY). Immediately before analysis of the cells on the flow cytometer, propidium iodide nuclear staining solution (PI, Serva) was added to the cells and the suspension was filtered through 46 µm nylon mesh. Cells were analysed on an EPICS 750 Series dual laser flow cytometer (Coulter Electronics, Hialeah, FL). A gate was set using the PI peak so that only single, nucleated cells were analysed. The percentage of LH-positive cells was determined from the plot of forward angle light scatter versus fluorescein-isothiocyanate fluorescence.

Radioimmunoassay

Concentrations of bovine LH in plasma, culture media and cell culture extracts were measured using the procedure

Table 1. Mass of bovine pars distalis and pars tuberalis, and cell yields following dispersion

Parameter	Pars distalis			Pars tuberalis		
	< 1	12	42	< 1	12	42
Mass (mg)	435 ± 67[a]	602 ± 79[a]	1488 ± 135[b]	58 ± 18[a]	56 ± 17[a]	130 ± 27[b]
Total cell yield (× 10^{-6})	47 ± 5[a]	62 ± 6[a]	100 ± 12[b]	3.5 ± 0.5[a]	4.2 ± 0.4[a]	3.0 ± 0.4[a]

All values are means ± SEM.
Means with different superscripts within rows for the pars distalis or the pars tuberalis are significantly different ($P < 0.05$).

described by Deaver and Peters (1988). The LH reference preparation used was USDA-bLH-B5, and the LH antisera was RAOLH TEA no. 35. The sensitivity of the assay was 25 pg per tube and the intra-assay and interassay coefficients of variation were each less than 10%.

Statistical analysis

The PULSAR program (Merriam and Wachter, 1982) was used to determine basal concentrations of LH and pulse frequency and pulse amplitude. Data were analysed using the Statistical Analysis System (SAS, SAS Institute, Cary, NC) General Linear Model (GLM) procedure for a completely random design; Duncan's multiple range test was used when the effect of age was significant.

Results

Pituitary masses and immunocytochemistry

The mass of the pars distalis was similar at 1 and 12 weeks but increased ($P < 0.05$) between 12 and 42 weeks of age (Table 1). In addition, the total number of cells obtained after dispersion increased between 12 and 42 weeks (Table 1), while cell yield per gram of tissue remained the same (data not shown). The percentage of cells from the pars distalis that were immunopositive for LH averaged 8.4% ± 0.8% and did not change with age (data not shown). However, since the number of cells recovered increased between 12 and 42 weeks, so did the total number of gonadotrophs recovered. The mass of the pars tuberalis also increased with age between 12 and 42 weeks ($P < 0.05$). However, in contrast to the pars distalis, the total number of cells recovered from the pars tuberalis did not change with age and averaged 3.57 ± 0.26 × 10^6.

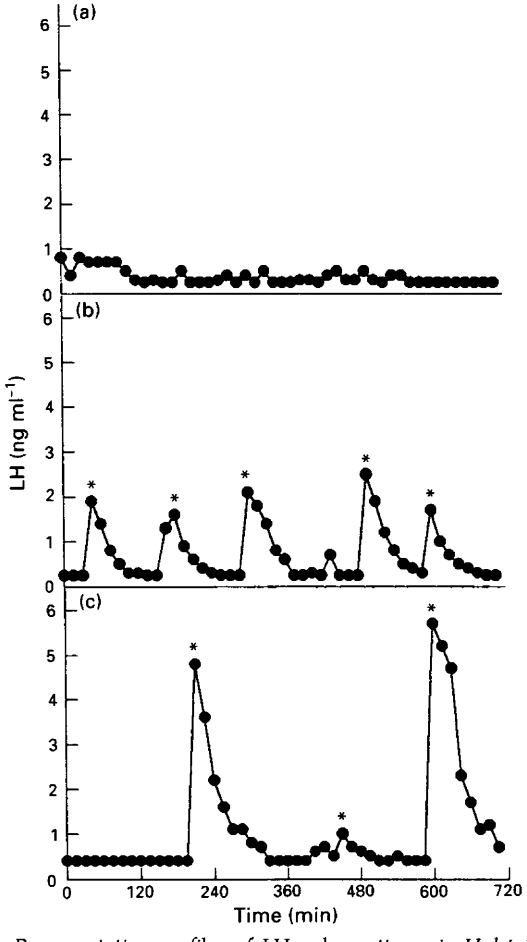

Fig. 1. Representative profiles of LH pulse patterns in Holstein bull calves at (a) < 1, (b) 12 and (c) 42 weeks of age. Blood samples were collected from calves every 15 min for 12 h using indwelling jugular catheters. The Pulsar algorithm (Merriam and Wachter, 1982) was used to detect pulses of LH, which are denoted by asterisks.

Secretion of LH in vivo

As expected, the pulse frequency of LH and its average concentration increased ($P < 0.01$) between < 1 and 12 weeks of age (Figs 1 and 2). Between 12 and 42 weeks the basal concentration of LH increased ($P < 0.01$) while LH pulse frequency declined. Pulse amplitude of LH did not differ between 12 and 42 weeks; too few pulses were observed in calves < 1 week old to determine LH pulse amplitude accurately at this age.

Secretion of LH by the pars distalis in vitro

The intracellular content of LH immediately after dispersion changed with age ($P < 0.01$), and was greatest at 12 weeks (Fig. 3). At both < 1 and 12 weeks the amount of LH present after 18 and 72 h of culture was similar to the initial content. In contrast, the total content of LH after 18 and 72 h in culture from pars distalis cells obtained at 42 weeks of age was higher ($P < 0.05$) than the initial content of LH, indicating a net

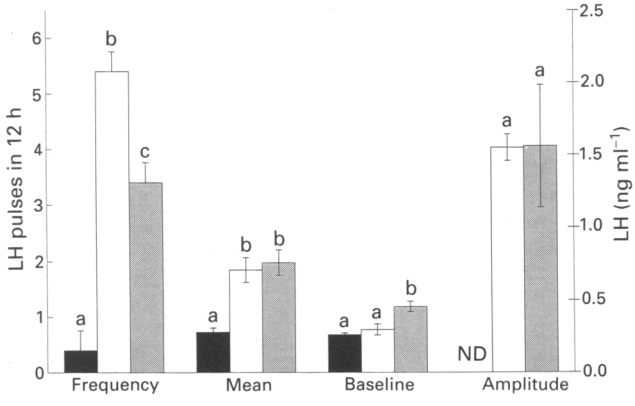

Fig. 2. Age-related changes in LH pulse frequency and amplitude, and mean and baseline concentrations of LH in Holstein bull calves. Values shown are means ± SEM at (■) < 1, (□) 12 and (▩) 42 weeks of age. Means with different superscripts are significantly different ($P < 0.01$). The mean concentration of LH increased between < 1 and 12 weeks of age. This initial increase was associated with a marked increase in LH pulse frequency. While there was no increase in LH pulse amplitude between 12 and 42 weeks, the baseline concentration of LH was greatest at 42 weeks of age.

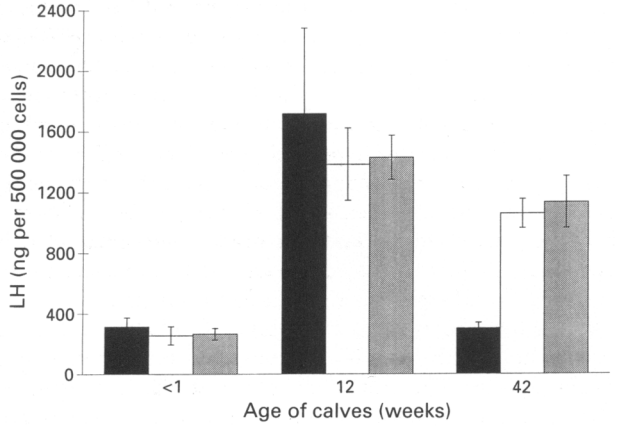

Fig. 3. Amount of LH in pars distalis from Holstein bull calves immediately after cell dispersion, and after 18 and 72 h in culture. Values shown are means ± SEM of (■) initial and (□) total (media plus intracellular) content after 18 and (▩) 72 h of culture. Anterior pituitary glands were removed from Holstein bull calves at < 1, 12 and 42 weeks of age. The pars distalis was enzymatically dispersed into a suspension of single cells. Cells were then (i) extracted with 0.01 mol $NaHCO_3$ l^{-1} or (ii) cultured for 18 and 72 h in α-MEM (α-minimum essential medium) with 2.5% homologous calf serum. The initial LH content was greatest at 12 weeks of age. The content of LH in the cultures after 18 and 72 h changed with age, and was greatest in cultures of cells obtained from calves at 12 weeks of age. Only at 42 weeks was the total content of LH after culture greater ($P < 0.05$) than the initial LH content.

synthesis of LH. The release of LH *in vitro* by pars distalis cells during 18 and 72 h in culture is shown in Fig. 4. The amount of LH released into media at < 1 and 12 weeks of culture was related to the initial LH cell content. There was a change in the percentage of LH released, with 37% and 50% of the total being in the media of < 1 and 12 weeks, respectively. In

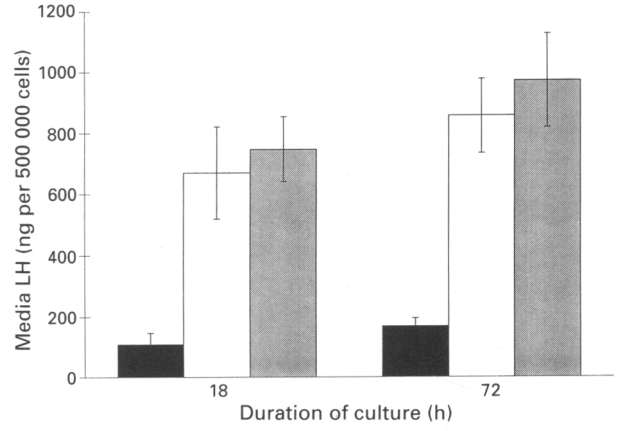

Fig. 4. Release of LH into media by pars distalis cells in Holstein bull calves after 18 and 72 h of culture. Values shown are means ± SEM at (■) < 1, (□) 12 and (▩) 42 weeks of age. After both 18 and 72 h in culture, LH concentration in media was lower in cultures obtained from calves at < 1 week of age when compared with cultures prepared from calves at 12 and 42 weeks of age.

cultures from 42-week-old calves, the LH content of media exceeded that initially present and was about 60% of the total present at the end of the culture.

Secretion of LH by the pars tuberalis in vitro

In preliminary experiments it was found that immediately after cell dispersion, the amount of LH in pars tuberalis cells was consistently below the limit of detection (data not shown). In this study, LH was detectable in media and cell extracts after culture. The combined content of LH in media and cell extracts (Table 2) did not increase between 18 and 72 h. There was a tendency for the total amount of LH, and that released into media, to increase with age; these differences were not statistically significant.

Discussion

Several observations were made in this study that further our understanding of age-related changes in the hypothalamic–pituitary–gonadal axis of the bull. First, although the percentage of cells from the pars distalis did not change with age, the total number of LH-positive cells recovered did increase markedly with age. Second, the pars distalis cells derived from the 12-week-old calves had the highest initial intracellular content of LH. Third, a net synthesis of LH occurred during culture of pars distalis cells obtained at 42 weeks of age. Finally, cells obtained from the pars tuberalis from all ages could synthesize LH in culture. However, their ability to produce LH did not seem to vary with age, making their function *in vitro* distinct from cells obtained from the pars distalis.

The increase in the frequency of pulsatile LH release in bull calves during the transition from the infantile to juvenile stage of development is well documented (Amann and Walker, 1983; Amann *et al.*, 1986). This increase in LH release is critical for the normal initiation of testicular growth and development

Table 2. Amount of bovine LH (ng per 500 000 cells) in media and cell extracts of pars tuberalis after 18 and 72 h of culture

	Media			Total		
Time (h)	< 1	12	42	< 1	12	42
18	98.7 ± 19	242 ± 54	399 ± 134	214 ± 32	432 ± 116	496 ± 159
72	168 ± 40	299 ± 87	547 ± 195	249 ± 32	398 ± 100	618 ± 217

All values are means ± SEM.

(Deaver *et al.*, 1988). On the basis of the results of this study, it is likely that the increased capacity of gonadotrophs to secrete LH contributes significantly to the increase in circulating LH. Neither the mass nor cell yield per gram of tissue changed between < 1 and 12 weeks of age, and the percentage of the dispersed pituitary cell population that stained positively for LH remained constant. However, the initial content of LH in pars distalis cells increased by more than a factor of five. It is widely accepted that GnRH plays an important role in biosynthesis of LH (Wildt *et al.*, 1981). Pulsatile release of GnRH is necessary for the expression of the mRNA encoding the α and β subunits of LH in the anterior pituitary (Hamernik *et al.*, 1986). The dramatic increase in the amount of LH produced in the pars distalis observed in this study can be accounted for by increases in both the frequency of GnRH release (Rodriguez and Wise, 1989) and in the concentration of pituitary GnRH receptors (Amann *et al.*, 1986).

In general, the age-related changes in LH secretion *in vivo* seen in the present study are in accordance with data reported by others (MacMillan and Hafs, 1968; Rawlings *et al.*, 1978; Lacroix and Pelletier, 1979; McCarthy *et al.*, 1979; Amann, 1983; Deaver and Peters, 1988; MacDonald *et al.*, 1990). In this study, we did not observe an increase in LH pulse amplitude between 12 and 42 weeks of age as we have previously reported (Deaver and Peters, 1988). However, since the volume of plasma increases with age, and the half-life of LH is not thought to change substantially, the amount of LH released with each pulse must be greater in older calves to maintain LH pulse amplitude. Increased pituitary sensitivity to exogenous GnRH during sexual development in bulls has been reported (Bass *et al.*, 1979; Chantaraprateep and Thibier, 1979), and may contribute to greater secretion rates in older calves. In addition, the mass of the pars distalis and the total number of gonadotrophs recovered increased significantly with age. Thus, an age-related hyperplasia of the gonadotroph population is probably important for maintaining adequate circulating concentrations of LH for spermatogenesis and continued testicular growth after puberty. A similar situation may occur in postpartum ewes, in which increased LH pulse amplitude has been associated with an increased number of gonadotrophs (Wise *et al.*, 1986).

The 'gonadostat' hypothesis proposed by Ramirez and McCann (1963) states that gonadal oestrogens inhibit the hypothalamic centres controlling the secretion of LH before puberty. Evidence supporting this hypothesis has been found in both sheep and cattle. Castration of infantile animals results in an early onset of pulsatile secretion of LH, and the ability of oestradiol to inhibit the secretion of LH decreases with age in lambs (Foster and Ryan, 1979) and heifers (Day *et al.*, 1984). Effects of testosterone and androstenedione on LH secretion are thought to be mediated by oestradiol in ruminants (Schanbacher *et al.*, 1983). Hypothalamic tissue has been shown to have the ability to convert androstenedione to oestradiol in rams (D'Occhio *et al.*, 1983) and in other species including humans, rhesus monkeys, rabbits, rats and mice (Naftolin *et al.*, 1975). Amann and co-workers (1986) found that the concentration of oestradiol in plasma and the number of hypothalamic receptors for oestradiol decrease before the initial increase in pulsatile secretion of LH in Holstein bull calves. Thus, the hypothalamus is probably an important site of negative feedback by testosterone and oestradiol in calves. Evidence to support this hypothesis was given by Deaver *et al.* (1988), who found that immature bulls implanted with oestradiol have an increased density of staining of GnRH fibres in the stalk median eminence. These results were interpreted by the authors to indicate that exogenous oestradiol decreases the release of GnRH from the stalk median eminence.

The above observations on negative feedback effects could explain, in part, the results obtained from the culture of pars distalis cells *in vitro* from bull calves at different ages. An interesting finding was that a net synthesis of LH was observed in the pars distalis cultures using cells derived from 42-week-old calves. This may represent a release from testosterone-mediated negative feedback that is present *in vivo* at this time (Deaver and Peters, 1988). Alternatively, it is possible that in older calves, gonadotrophs have a higher rate of LH synthesis, but LH content is lower because a greater proportion of synthesized LH is released following each endogenous GnRH discharge. The accumulation of LH during the first 18 h of culture may reflect this higher rate of LH synthesis. It is evident that the increased synthesis and release of LH observed in cultured cells from these older animals was not dependent on the continued presence of GnRH.

The pars tuberalis represents a component of the adenohypophysis, 5–10% of which constitutes gonadotrophs and which possess few other secretory cell types found in the pars distalis based on immunocytochemistry (Gross *et al.*, 1984). Recently, the pars tuberalis has been implicated as a potential target site for melatonin in regulating seasonal reproduction (De Reviers *et al.*, 1989; Morgan *et al.*, 1989), and it has been suggested that the pars tuberalis may secrete a peptide that modulates pars distalis function (Wittkowski *et al.*, 1992). When

undertaking preliminary studies for the study reported here, LH could not be detected in pars tuberalis cell extracts immediately after dispersion. However, in agreement with others (Gross *et al.*, 1984) we found that (i) LH can be detected in tissue homogenates of the pars tuberalis prepared immediately after tissue collection and (ii) LH is released during perifusion of the bovine pars tuberalis. Consequently, we consider that the initial content of LH is lost from pars tuberalis cells during the dispersion procedure. This may be caused by the release of GnRH from the stalk median eminence (which is present during the dispersion), or by a high rate of release with little storage, since most of the cells of the pars tuberalis are agranular or sparsely granulated (Morgan *et al.*, 1991). On the basis of the results from this study, it appears that gonadotrophs from the pars tuberalis differ from those obtained from the pars distalis. Significant amounts of LH were present after 18 h of culture of pars tuberalis cells, indicating the synthesis of LH during culture at all ages. No age-related changes occurred in the ability of pars tuberalis cells to produce LH during culture. However, it should be noted that dispersed, cultured pars tuberalis cells might function differently from those *in situ*. The role that the pars tuberalis plays, if any, in regulating the onset of puberty in cattle is not clear and requires further investigation.

In conclusion, we report here that age-related differences in the numbers and secretory activity of gonadotrophs in the bull calves appear to contribute to age-related differences in concentrations of LH in plasma. Different changes in the synthesis and release of LH from pars distalis and pars tuberalis cells occurred *in vitro*, suggesting that there is differential regulation in these regions in bull calves.

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