Opioidergic, dopaminergic and adrenergic regulation of LH secretion in prepubertal heifers

A. Honaramooz, R. K. Chandolia*, A. P. Beard[†] and N.C. Rawlings[‡]

Department of Veterinary Physiological Sciences, Western College of Veterinary Medicine, 52 Campus Drive, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5B4, Canada

Studies have shown inhibitory effects of endogenous opioids on LH secretion in early post-natal heifers. However, it is not clear whether these effects change during the rest of the prepubertal period or whether the inhibitory influences on the GnRH neurones are direct or by way of other neuronal systems. Two experiments were performed in heifer calves to study the developmental patterns of opioidergic, dopaminergic and adrenergic regulation of LH and the possible interactions between opioids and dopaminergic and adrenergic neuronal systems, in the regulation of LH secretion. In Expt 1 four groups each of five heifer calves were used. Blood samples were taken every 15 min for 10 h and each calf received one of the following treatments as a single injection at 4, 14, 24, 36 and 48 weeks of age: (i) naloxone (opioid antagonist, 1 mg kg⁻¹, i.v.); (ii) sulpiride (dopamine D2 antagonist, 0.59 mg kg⁻¹, s.c.); (iii) naloxone and sulpiride combined; or (iv) vehicle (control group). Treatments began after the first blood sample was taken. The design of Expt 2 was similar; a separate group of heifer calves was assigned to receive one of the following treatments as a single injection at 4, 14, 24, 36 and 48 weeks of age: (i) naloxone; (ii) phenoxybenzamine (an α -adrenoreceptor blocker, 0.8 mg kg⁻¹, i.v.); (iii) naloxone and phenoxybenzamine; (iv) or vehicle. Results from Expt 1 showed that the maximum concentration of LH and the number of calves responding to treatments with an LH pulse was higher in the first hour after treatments at 36 and 48 weeks of age in the naloxone group compared with the control or sulpiride groups ($P < 0.05$). These values in the naloxone group also increased over time and were greatest at 48 weeks of age ($P < 0.05$). In heifers given naloxone + sulpiride treatment at 36 and 48 weeks of age, maximum concentrations of LH in the first hour after treatment did not differ from the naloxone and control groups. In Expt 2, at 36 and 48 weeks of age, treatment with naloxone with or without phenoxybenzamine resulted in higher concentrations of LH than in the controls ($P < 0.05$). No pulses were seen over the first hour of treatment at 36 and 48 weeks of age in heifers treated with phenoxybenzamine. The 10 h periods of blood sampling at 48 weeks of age revealed that phenoxybenzamine alone suppressed LH pulse frequency and mean serum concentrations of LH compared with the control group ($P < 0.05$). It was concluded that a strong or more acute inhibition of LH secretion by endogenous opioids developed in mid- to late prepubertal heifers, or alternatively, that removal of opioidergic inhibition at the GnRH neurone unmasked stimulatory inputs that were greater in heifers close to first ovulation. Since sulpiride appeared to negate in part the effects of naloxone on LH release, the suppressive effects of opioids could be exerted in part through the inhibition or blocking of a stimulatory dopaminergic system. α -Adrenergic neuronal systems have stimulatory effects on LH release, especially during the late prepubertal period, but do not appear to mediate opioidergic inhibition of LH secretion in prepubertal heifer calves.

*Present address: Department of Gynaecology and Obstetrics, College of Veterinary Sciences, CCS Haryana Agricultural University, Hisar, 125004 India.

[†]Present address: Department of Agriculture, University of Newcastle, Newcastle NE1 7RU, UK.

[‡]Correspondence.

Received 25 October 1999.

Introduction

In beef heifers in Saskatchewan, first ovulation occurs at about 56 weeks of age (Evans *et al.*, 1992; Honaramooz *et al.*, 1998). During the prepubertal period in heifer calves, mean circulating concentrations of LH are low, but LH pulse amplitude may increase transiently between 10 and 22 weeks of age (Evans *et al.*, 1992). It is only during the last few weeks before first ovulation that an increase in LH pulse frequency is observed, resulting in an increase in mean circulating concentrations of LH (see Kinder *et al.*, 1987, 1995). There is evidence that the pituitary gland (Barnes *et al.*, 1980) and gonads (Onuman *et al.*, 1970; Seidel *et al.*, 1971) are capable of functional activity early in life in heifer calves. Therefore, increased hypothalamic GnRH secretion would appear to cause the early prepubertal increase in LH secretion (pulse amplitude, Evans *et al.*, 1992) and some final maturation or regulatory change at the hypothalamus probably leads to the increase in gonadotrophic drive (increased LH pulse frequency) before first ovulation (Dyer *et al.*, 1990).

In prepubertal heifers, the neuroendocrine mechanisms controlling the temporal pattern of circulating concentrations of LH are poorly understood. It has been suggested that in many species endogenous opioid peptides function as neurotransmitters in the hypothalamus, acting directly or indirectly on GnRH-producing neurones to decrease their secretion and thereby reduce the pituitary release of LH during the prepubertal period (see Brooks *et al.*, 1986; Haynes *et al.*, 1989; Barb *et al.*, 1991; Cosgrove *et al.*, 1993). In heifer calves, in the early post-natal period, inhibition of endogenous opioid peptides can result in increased LH release (Evans *et al.*, 1992). It is thought that a powerful gonadal oestrogen negative feedback is the main suppressive factor of LH secretion in the early prepubertal heifer (see Kinder *et al.*, 1987, 1995). Endogenous opioids might be involved in conveying this gonadal suppression (Wolfe *et al.*, 1991), but these effects decrease as heifers approach the expected age of puberty (Wolfe *et al.*, 1992). Chronic administration of naloxone (an endogenous opioid peptide antagonist) in neonatal female rats induced precocious puberty and it is believed that the release of an endogenous opioid peptide 'brake' may give rise to the gonadotrophic drive that occurs in the immediate prepubertal period (Sirinathsinghji *et al.*, 1985). In heifers, there are no reports of studies examining the role of endogenous opioid peptides in regulation of LH secretion in the entire prepubertal period (from birth to puberty).

It has been suggested that endogenous opioid peptide neurones may modulate LH secretion by way of, or in concert with, catecholaminergic neuronal pathways to the hypothalamus (see Kalra and Kalra, 1983; Barraclough *et al.*, 1984; Haynes *et al.*, 1989; Przekop and Tomaszewska, 1996). The developmental role of dopaminergic neuronal systems on the control of LH secretion in heifer calves is unknown. In bull calves, dopamine concentrations in the anterior hypothalamic-preoptic area increase two- to threefold between 5 and 10 weeks of age and it was proposed that they might influence the development of pulsatile LH release in post-natal bull calves (Rodriguez *et al.*, 1993). Chandolia *et al.* (1997) administered antagonists of endogenous opioid

peptides and dopamine to 6-, 14- and 24-week-old bull calves and demonstrated that a dopaminergic drive for LH secretion occurred at 24 weeks of age and that endogenous opioid peptide inhibition of LH release was mainly due to the inhibition of a dopamine drive at 24 weeks of age.

There is no consensus over the role of adrenergic neuronal systems on LH release. Experiments *in vitro* and *in vivo* in rats favour a stimulatory role for noradrenaline (see Barraclough and Wise, 1982; Ojeda *et al.*, 1982; Ramirez *et al.*, 1984; Adams and Steiner, 1988). In ruminants, most of the limited information has been obtained from sheep and the results indicate that stimulatory or inhibitory effects of adrenergic systems on LH release depend on the endocrine status of the ewe (see Dailey *et al.*, 1987). However, sheep are remarkably seasonal in their reproductive patterns and most neurotransmitter research has been directed toward understanding seasonality (see Dailey *et al.*, 1987). In prepubertal beef heifers, the response of LH secretion to a GnRH challenge was decreased after administration of noradrenaline, indicating an inhibitory role for noradrenaline on LH release at the pituitary (Hardin and Randel, 1983). Studies in immature and mature rats indicate that endogenous opioids affect, or interact with, adrenergic neuronal systems to alter LH secretion (Martensz *et al.*, 1990; Kim *et al.*, 1991; Nishihara *et al.*, 1991; Brann *et al.*, 1992; Smith and Gallo, 1997).

The aim of the present study was to examine whether endogenous opioid peptides, dopamine or noradrenaline neuronal systems or the interactions between endogenous opioid peptides and dopamine or noradrenaline were involved in the regulation of the temporal pattern of LH secretion and events leading to the first ovulation in heifer calves. The hypothesis was that the effects of each of the antagonists for endogenous opioid peptides, dopamine or noradrenaline would vary with age and that the inhibitory effects of endogenous opioid peptides would be changed with concomitant blockage of dopamine or noradrenaline systems.

Materials and Methods

Animals

Spring-born age-matched (± 5 days) Hereford heifer calves were raised in a beef management system; calves were suckled at pasture until weaning at 28 weeks of age, from which time they were maintained in paddocks and were provided with brome-alfalfa hay, water and a ground concentrate ration (Evans *et al.*, 1992; Honaramooz *et al.*, 1998). Body weight was recorded every 2 weeks from birth until puberty. Animals were treated and handled according to the guidelines of the Canada Council of Animal Care.

Experimental design and treatments

Experiment 1. Twenty calves were randomly assigned to four groups of five animals each. At 4, 14, 24, 36 and 48 weeks of age and after collecting a single blood sample, heifers

received one of the following treatments: (i) 1 mg kg⁻¹ body weight i.v. bolus injection of naloxone hydrochloride (opioid antagonist; Research Biochemicals International, Natick, MA) dissolved in saline; (ii) 0.59 mg kg⁻¹ body weight s.c. injection of (–)-sulpiride (dopamine D2-antagonist; Sigma Chemical Co., St Louis, MO) dissolved in saline containing 0.01 mol NaOH l⁻¹; (iii) naloxone and sulpiride administered as described above; or (iv) vehicle (control group). After 48 weeks of age, animals started to reach puberty and although the remaining non-pubertal heifers were treated at 52 and 56 weeks of age, the number of animals was insufficient for statistical comparisons (control $n = 3$ and 3; naloxone $n = 2$ and 2; sulpiride $n = 1$ and 1; and naloxone + sulpiride $n = 3$ and 2, at 52 and 56 weeks of age, respectively); therefore, data up to 48 weeks of age are presented. Naloxone has a half-life of 1 h in plasma (Reisine and Pasternak, 1995) and the half-life of sulpiride is about 7 h (Bres and Bressolle, 1991). Therefore the aim was to examine the acute response (1 h) of LH secretion to the treatments as well as a long-term (10 h) response to sulpiride injection. The doses of naloxone and sulpiride were based on results from experiments in cattle and sheep (Evans *et al.*, 1992; Tortonese and Lincoln, 1994).

At each treatment period (4, 14, 24, 36 and 48 weeks of age), blood samples (4–5 ml) were collected through jugular catheters every 15 min for 10 h (08:00 h to 18:00 h) to characterize the pulsatile pattern of circulating concentrations of LH. At 1 day before each period of treatment, calves were catheterized using single lumen PVC tubing with internal and external diameters of 1.0 mm and 1.5 mm, respectively (Critchley Electrical Products Pty Ltd, Silverwater BC, NSW). During blood sampling, calves were housed loose in pens in a barn and hay and water were provided. Before weaning, cows were haltered with their calves. Blood samples were allowed to clot for 12–18 h at room temperature, the clots were removed and serum was centrifuged at 1500 g for 15 min. The serum was poured off and stored at -20°C until analysed.

Starting at 48 weeks of age, jugular blood samples were drawn two to three times a week into sodium heparinized vacutainers (10 ml; Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) and centrifuged within 30 min of collection, to determine age at puberty. Plasma was aspirated and stored at -20°C until analysis. Puberty was defined as the age at which plasma concentrations of progesterone first exceeded 1.0 ng ml⁻¹, as an indication of first ovulation.

Experiment 2. Twenty calves were randomly assigned to four groups each of five heifers. After collecting a single blood sample at 4, 14, 24, 36 and 48 weeks of age, heifers were injected with one of the following treatments: (i) 1 mg kg⁻¹ body weight i.v. bolus injection of naloxone; (ii) 0.8 mg kg⁻¹ body weight i.v. bolus injection of phenoxybenzamine hydrochloride (α -adrenoceptor blocker; Research Biochemicals International) dissolved in ethanol-propylene glycol; (iii) naloxone and phenoxybenzamine administered as described above; or (iv) vehicle (control group). After 48 weeks of age, some animals had reached puberty and although the remaining non-pubertal heifers were treated at 52 and 56 weeks of age, the number of animals was insufficient for statistical comparisons (control $n = 3$ and 2; naloxone $n = 2$

and 2; phenoxybenzamine $n = 2$ and 1; and naloxone + phenoxybenzamine $n = 3$ and 1, at 52 and 56 weeks of age, respectively). Therefore, data up to 48 weeks of age are presented. Phenoxybenzamine has a half-life of about 24 h (Hoffman and Lefkowitz, 1995) and therefore in addition to studying the acute response of LH secretion to the treatments, the long-term response of LH secretion to the phenoxybenzamine treatment was also considered. The dose of phenoxybenzamine was based on results from an experiment in sheep (Meyer and Goodman, 1985).

Procedures for periods of frequent blood sampling, and sampling for the detection of first ovulation as well as blood handling, were performed according to the design described for Expt 1.

Radioimmunoassays

Serum concentrations of LH were determined in double antibody radioimmunoassays (Rawlings *et al.*, 1984; Evans *et al.*, 1992). LH concentrations are expressed in terms of NIDDK-bLH4 and the sensitivity of the assay was 0.1 ng ml⁻¹. The range of the standard curve was from 0.06 to 8.00 ng ml⁻¹, and the intra- and interassay coefficients of variation were 9 and 10% (mean 0.28 ng ml⁻¹) and 7 and 9% (mean 0.86 ng ml⁻¹), respectively.

Detection of LH pulses and statistical analysis

The episodic patterns of circulating concentrations of LH in blood samples collected during each treatment period were determined using the PC-Pulsar program (J. Gitzen and V. Ramirez, University of Illinois, IL). These patterns are expressed in terms of mean and basal serum concentrations of LH and LH pulse frequency and amplitude. Pulses were identified using standard deviation criteria of height (G values) and duration (Merriam and Watcher, 1982). The G values were as reported by Honaramooz *et al.* (1998). The basal circulating concentrations of LH excluded all hormone values included in the pulses.

Throughout the statistical analysis, the data for the two experiments were analysed similarly but separately. Since naloxone has a short half-life (1 h), but sulpiride and phenoxybenzamine have longer half-lives (7 and 24 h, respectively), the short-term (acute) response of LH secretion to the treatments (first hour of sampling) was compared for all groups. However, among groups, comparisons of serum concentrations of LH during the 10 h periods of sampling were considered for the control and sulpiride groups (Expt 1) or the control and phenoxybenzamine groups (Expt 2) for effects of treatment, age and interactions using repeated measures ANOVA (RM-ANOVA, Sigma Stat for Windows Version 1.0, Jandel Corporation, San Rafael CA). The number of animals with an LH pulse (detected by the Pulsar analysis) during the first hour of sampling after starting treatments was compared among groups at each age and among ages within groups, using chi-squared analysis (True Epistat: Epistat Services, Richardson, TX) to test the acute LH response to different treatments. The acute response of LH

secretion also was examined by comparing the peak concentration of LH pulses during the first hour of treatment periods for effects of treatment, age and their interactions, using repeated measures ANOVA (RM-ANOVA, Sigma Stat). In this comparison, when there were no LH pulses, the average LH concentrations during the 1 h period were taken. In all analyses, if the main effects were significant, Student–Newman–Keuls test for least significant differences ($P < 0.05$) was used as the post-ANOVA test. All data are presented as mean $\pm$ standard error of mean (SEM).

Results

Experiment 1

Body weight and age at puberty. Body weight at birth and at puberty (first ovulation) did not differ among groups (38 ± 2 , 40 ± 2 , 39 ± 1 and 43 ± 3 kg, and 407 ± 20 , 366 ± 11 , 358 ± 14 and 345 ± 24 kg in control, naloxone, sulpiride and naloxone + sulpiride groups, respectively). Age at puberty was not affected by any of the treatments (58 ± 3 , 54 ± 3 , 53 ± 3 and 57 ± 3 weeks).

Effects of treatments on LH secretion. Maximum concentrations of LH released within 1 h of treatments are shown (Fig. 1a). At 36 and 48 weeks of age, naloxone treatment alone increased LH release compared with control calves or calves given sulpiride ($P < 0.05$). At these ages, calves in the naloxone + sulpiride group did not differ from the control or naloxone-treated heifers. The maximum LH response to naloxone also increased over time; the greatest response was seen at 36 and 48 weeks of age ($P < 0.05$).

The number of calves responding with an LH pulse within 1 h of injection of naloxone increased with age and all heifers responded at 36 and 48 weeks of age ($P < 0.05$, Fig. 2a). The number of calves with an LH pulse within 1 h of treatment was higher in the naloxone-treated calves than in the control and sulpiride groups at 36 weeks of age and than in the sulpiride group at 48 weeks of age ($P < 0.05$, Fig. 2a). In calves given naloxone + sulpiride at 36 and 48 weeks of age, maximum LH concentrations and the number of calves having an LH pulse within 1 h of treatment were intermediate to the naloxone and control calves (that is, not different from naloxone or control groups).

On the basis of the 10 h sampling periods in control and sulpiride-treated animals, the two groups did not differ at any age, but mean concentrations of LH in the sulpiride group was highest at 48 weeks of age ($P < 0.05$) and LH pulse frequency in control heifers also changed over time and was highest at 48 weeks of age ($P < 0.05$, Fig. 3). Basal serum concentrations of LH and LH pulse amplitude did not differ between the two groups at any age or over time within each group.

Experiment 2

Body weight and age at puberty. Body weight at birth and at puberty (first ovulation) did not differ among the treatment groups (37 ± 2 , 41 ± 3 , 41 ± 2 and 40 ± 2 kg, and 410 ± 24 ,

389 ± 14 , 398 ± 19 and 386 ± 15 kg in the control, naloxone, phenoxybenzamine and naloxone + phenoxybenzamine groups, respectively). Age at puberty did not differ among groups (56 ± 2 , 53 ± 3 , 55 ± 1 and 56 ± 2 weeks, respectively).

Effects of treatments on LH secretion. At 36 and 48 weeks of age, the LH maximum response was greater in both the naloxone and naloxone + phenoxybenzamine groups than in the control or phenoxybenzamine groups ($P < 0.05$, Fig. 1b). The maximum LH response to naloxone was greater at 36 and 48 weeks age than at 4 and 24 weeks of age ($P < 0.05$, Fig. 1b). The response to naloxone + phenoxybenzamine also changed with time and was greater at 48 weeks of age than at 4, 14 or 24 weeks of age ($P < 0.05$, Fig. 1b).

The number of calves with an LH pulse within 1 h of treatment was higher in the naloxone-treated calves than in the control and phenoxybenzamine groups at 36 weeks of age ($P < 0.05$, Fig. 2b). At 48 weeks of age, more calves in the naloxone and naloxone + phenoxybenzamine groups had a pulse than in the phenoxybenzamine group ($P < 0.05$, Fig. 2b). The number of calves responding with an LH pulse within 1 h of treatment changed over time and all heifers responded to naloxone at 36 weeks of age and to naloxone + phenoxybenzamine at 48 weeks of age ($P < 0.05$, Fig. 2b).

On the basis of the 10 h sampling periods, LH pulse frequency and mean LH concentrations were lower in phenoxybenzamine-treated calves compared with control calves at 48 weeks of age ($P < 0.05$, Fig. 4). In control calves, mean serum concentrations of LH changed over time and were highest at 48 weeks of age ($P < 0.05$, Fig. 4). LH pulse frequency also changed over time and was highest at 48 weeks of age in both the control and phenoxybenzamine groups ($P < 0.05$, Fig. 4). Basal serum concentrations of LH and LH pulse amplitude did not differ between the two groups at any age or over time within each group.

Discussion

Comparing the first hour of the periods of frequent blood sampling, it appears that the acute response to naloxone in both experiments developed and was more consistent as the calves aged. In another study, when heifer calves were examined up to 32 weeks of age (Evans *et al.*, 1992), significant increases in LH pulse frequency were seen only after naloxone administration once an hour over a 12 h period in heifer calves at 4 and 24 weeks of age, with non-significant numerical increases at 12 and 18 weeks of age (Evans *et al.*, 1992). This indicates that although endogenous opioid peptides may weakly suppress LH secretion in calves in the early post-natal period, this inhibitory effect becomes much stronger or more acute as heifers age, reaching a maximum in the mid- to late prepubertal period. Alternatively, removal of direct opioidergic inhibition of GnRH neurones may have unmasked stimulatory neuronal inputs that increase with age.

The question remains as to why and how an increase in LH pulse frequency can occur before the first ovulation in the presence of the increasing potency of endogenous opioid

Fig. 1. Maximum serum concentrations of LH (mean + SEM) during 1 h after treatment of heifer calves with (a) saline (control, □), naloxone (▨) sulpiride (▤) or naloxone + sulpiride (■) or (b) saline (control, □), naloxone (▨), phenoxybenzamine (▤) or naloxone + phenoxybenzamine (■) at 4, 14, 24, 36 and 48 weeks of age. Values with different letters a, b (between treatments within ages) or x, y, z (between age within treatments) are significantly different ($P < 0.05$).

peptides. The first ovulation in heifers is preceded by a marked increase in LH pulse frequency (see Kinder *et al.*, 1987, 1995) and it has been suggested that a reduction in opioidergic suppression of LH secretion may occur in heifers immediately before the first ovulation (Wolfe *et al.*, 1992). If this is the case, the present results indicate that, in the mid- to late prepubertal period, a mechanism with the ability to control increases in LH pulse frequency is brought into play. Endogenous opioid peptides may serve as such a brake, applying some restraint to the prepubertal increase in LH pulse frequency. An endogenous opioid peptide brake on LH secretion, as suggested by Sirinathsinghi *et al.* (1985) in peripubertal rats, and its rapid withdrawal (Wolfe *et al.*, 1992) may enable the system to control the timing of first ovulation. It has been suggested that negative feedback by oestradiol may restrain LH secretion in prepubertal heifers and that a reduction in this effect may allow LH pulse frequency to increase to give the first ovulation (see Kinder *et*

al., 1987, 1995). This conclusion is consistent with the observation that the number of receptors for oestrogen in the hypothalamus decreases during the peripubertal period in heifers (Day *et al.*, 1987). It may be postulated that as the negative effect of oestradiol on LH release is diminished, inhibitory effects of endogenous opioid peptides become more apparent to fine tune the occurrence of the first ovulation. It has been demonstrated that endogenous opioid peptides mediate the effects of oestradiol in inhibiting LH secretion in peripubertal heifers (Wolfe *et al.*, 1991) and such effects decrease as heifers develop from prepubertal to peripubertal period (Wolfe *et al.*, 1992). However, all or part of the inhibitory effect of endogenous opioid peptides on LH secretion may be independent of oestradiol.

It is believed that the effect of endogenous opioid peptide inhibitors on LH release is due to their influence on GnRH neurones. Support for the possibility of direct trans-synaptic regulation of GnRH by endogenous opioid peptides was

Fig. 2. Number of calves responding with an LH pulse within 1 h of treatment with (a) saline (control, □), naloxone (▨), sulpiride (■) or naloxone + sulpiride (■) or (b) saline (control, □), naloxone (▨), phenoxybenzamine (■) or naloxone + phenoxybenzamine (■) at 4, 14, 24, 36 and 48 weeks of age. Values with different letters a, b (between treatments within ages) or x, y, z (between age within treatments) are significantly different ($P < 0.05$).

provided by studies showing the presence of synapses between endogenous opioid peptide-containing neurones and some GnRH neurones in female rats (Leranth *et al.*, 1988; Chen *et al.*, 1990) and monkeys (Thind and Goldsmith, 1988). However, the fact that endogenous opioid peptides may function directly upon GnRH neurones has been challenged by more recent evidence obtained from *in situ* hybridization, which showed that mRNA for endogenous opioid peptide receptors was present within rostral preoptic area cells but GnRH neurones did not seem to synthesize these mRNAs (Sannella and Petersen, 1997). Some pharmacological studies have shown that endogenous opioid peptides may affect GnRH release by acting within the mediobasal hypothalamus (Drouva *et al.*, 1981; Kalra, 1981). The amplitude of LH pulses induced by GnRH challenge in heifers did not significantly change with age when given once a month from birth to 9 months of age (Schams *et al.*, 1981). Therefore, the increasing responsiveness of LH secretion to administration of naloxone with age cannot be due to the higher sensitivity of the pituitary to the naloxone-stimulated GnRH release.

The present results do not show evidence of a direct dopamine drive for LH secretion as reported for 24-week-old bull calves (Chandolia *et al.*, 1997). A stimulatory role for dopamine is in contrast to the predominant view of dopamine as an inhibitor of LH release in sheep (Meyer and Goodman, 1985; Curlewis *et al.*, 1991; Tortonese and Lincoln, 1994; Tortonese, 1999). Almost all studies on effects of dopamine in female ruminants have been conducted in sheep and the results varied depending on the season, the dose administered and the physiological status of the animals (see Dailey *et al.*, 1987; Chomicka, 1992; Gallegos-Sanchez *et al.*, 1998). There is evidence for the presence of a synapse between dopamine and GnRH neurones in the ewe median eminence and for dopamine suppression of GnRH release from the median eminence *in vitro* (Kuljis and Advis, 1989). An inhibitory effect of dopamine on LH release has been described in anoestrous ewes, using dopamine receptor antagonists (Meyer and

Goodman, 1985; Curlewis *et al.*, 1991; Tortonese and Lincoln, 1994), chemical disruption (Thiery, 1991) or hypothalamic deafferentation (Przekop, 1978; Pau *et al.*, 1982) of dopamine systems. In ewes during the breeding season, administration of dopamine antagonist did not cause a change in LH release (Meyer and Goodman, 1985), indicating that dopamine systems are more active during anoestrus. However, there are reports of studies in which central (Przekop *et al.*, 1975) or systemic (Deaver and Daily, 1982) administration of dopamine did not influence LH release in anoestrous ewes or that dopamine had an inhibitory role in ewes during the breeding season (McNeilly, 1980; Deaver and Daily, 1983). An inhibitory effect of dopamine systems on LH release has also been shown during the peripubertal period in ewe lambs, but this effect appeared to become functional between 17 and 22 weeks of age (Brango *et al.*, 1990). Owing to the extreme effects of season on reproductive parameters in sheep, generalization of the results to cattle may not be suitable. For instance, cattle appear to be more similar to rats than sheep in the responses to naloxone during pubertal development, which might be due to their relative resistance to changes in photoperiod (Haynes *et al.*, 1989). In a similar way, endogenous opioid peptides seem to inhibit dopamine systems in rats (see below, Holtt and Bergmann, 1982; Locatelli *et al.*, 1983; Vermes *et al.*, 1984), whereas in sheep, interaction between endogenous opioid peptides and dopamine systems were reported to occur in the reverse order (that is, dopamine inhibits endogenous opioid peptide systems that control LH release) (Tortonese, 1999). However, even in a given species, interpretation of results is dependent upon the pharmacological agent used, the rate of administration, the dose used and the physiological state of the animal (Deaver and Daily, 1982).

In the present study, the addition of sulpiride, a dopamine receptor blocker, to the naloxone treatment decreased the effects of naloxone on LH in older heifer calves. It is interesting to compare the maximum LH response to naloxone and the combination treatment between the two

Fig. 3. Mean serum LH concentrations (a) and LH pulse frequency (mean + SEM) (b) in heifer calves treated with saline (control, □) or sulpiride (▨) for 10 h at 4, 14, 24, 36 and 48 weeks of age; blood samples were taken every 15 min. Values with different letters x, y (between age within treatments) are significantly different ($P < 0.05$).

Fig. 4. Mean serum LH concentrations (a) and LH pulse frequency (mean + SEM) (b) in heifer calves treated with saline (control, □) or phenoxybenzamine (▨) for 10 h at 4, 14, 24, 36 and 48 weeks of age; blood samples were taken every 15 min. Values with different letters a, b (between treatments within ages) or x, y (between age within treatments) are significantly different ($P < 0.05$).

experiments. At 36 and 48 weeks of age, the naloxone + sulpiride group did not differ from the control or naloxone group, whereas the naloxone + phenoxybenzamine and naloxone groups responded similarly. This finding indicates that the endogenous opioid peptide inhibition of LH release was exerted, to some extent, through the inhibition of dopamine neurones and, therefore, is an indirect effect on GnRH neurones. Alternatively, removal of endogenous opioid peptide inhibition may have exposed a stimulatory dopamine effect on the GnRH neurones; this explanation would concur with the lack of effect of sulpiride when given alone. Interaction of endogenous opioid peptides and dopamine neuronal systems in the regulation of LH release has been shown in rats (Rasmussen, 1991). In peripubertal bull calves, endogenous opioid peptide inhibition of LH secretion largely involved inhibition of a dopamine drive at 24 weeks of age, but not at earlier stages (Chandolia *et al.*, 1997). Although morphine treatment did not change the pattern of GnRH secretion in GT1-7 cells, and dopamine increased GnRH release, pretreatment with morphine suppressed the GnRH response to dopamine (Nazian *et al.*, 1994). However, in rams, a combination of dopamine agonist

and antagonist and an endogenous opioid peptide antagonist, revealed that, especially in the sexually inactive phase under long days, dopamine has a predominantly inhibitory effect on both GnRH and endogenous opioid peptide systems and that photoperiodic control of the reproductive cycle may modulate the interplay between dopamine and endogenous opioid peptides (Tortorese, 1999). The release of LH was unaffected by treating peripubertal ewe lambs with antagonists of either dopamine or endogenous opioid peptides; however, when the treatments were combined, LH release was significantly increased (Haynes *et al.*, 1989). There are no reports of studies of interactions of endogenous opioid peptides and dopamine neuronal systems in the regulation of LH secretion in peripubertal heifers.

Treatment with phenoxybenzamine alone decreased mean concentrations of LH and LH pulse frequency at 48 weeks of age compared with the control heifer calves. Within 1 h after treatments at 36 and 48 weeks of age, none of the heifers treated with phenoxybenzamine had an LH pulse. These results indicate a stimulatory role for α -adrenergic neuronal systems on LH release in peripubertal heifers, which perhaps

become more significant as the first ovulation approaches. Hardin and Randel (1983) demonstrated that adrenaline or noradrenaline injections in peripubertal beef heifers did not change the normal pattern of LH secretion, but reduced the magnitude of the serum LH response to GnRH challenge, indicating an inhibitory effect of adrenergic systems on LH release at the pituitary.

Interactions of opioidergic and adrenergic neuronal systems in the regulation of LH release have been shown in female rats (Nishihara *et al.*, 1991; Brann *et al.*, 1992; Smith and Gallo, 1997). In the present study, there was little evidence of an α -adrenergic mediation of the inhibitory effects of endogenous opioid peptides on LH secretion (that is blockage of the effects of naloxone by phenoxybenzamine). LH secretion in heifers given the combination of phenoxybenzamine and naloxone resembled those of naloxone-treated calves, particularly in older animals (48 weeks of age).

In conclusion, the present results indicate that an acute and strong inhibitory effect of endogenous opioids develops as heifer calves approach first ovulation. Alternatively, blocking opioidergic inhibition unmasks stimulatory neuronal influences to the GnRH neurone that increase as the heifer approaches first ovulation. Subsequent removal of this inhibition (Wolfe *et al.*, 1992) may fine tune the timing of first ovulation. Since sulpiride partly negated the effects of naloxone on LH release, the suppressive effects of opioids would appear to be exerted, in part, through the inhibition of a dopaminergic neuronal system or by way of a generalized inhibition of the GnRH neurones in the presence of other stimulatory inputs. α -Adrenergic neuronal systems have stimulatory effects on LH release, especially during the late prepubertal period before first ovulation, but do not appear to mediate opioidergic inhibition of LH secretion in prepubertal heifer calves.

The authors give special thanks to Susan Cook, Tim Hegan, Donna Waldbilling and Michelle Nelson for their excellent technical assistance, Bill Kerr and his staff for care and management of the animals, and NIDDK and USDA for the provision of purified hormones. This study was funded by the Natural Sciences and Engineering Research Council of Canada.

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