Fluctuation in responsiveness of LH and lack of responsiveness of FSH to prolonged infusion of morphine and naloxone in the ewe

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Summary. In ewes in the mid-luteal phase, LH pulse frequency ($P < 0.01$) and amplitude ($P < 0.05$) increased during a 24 h infusion of naloxone (0.5 mg/kg/h) compared to a 24 h infusion of vehicle (mean $\pm$ s.e.m.; 0.25 ± 0.03 vs 0.14 ± 0.01 pulses/h and 0.84 ± 0.08 vs 0.55 ± 0.08 ng/ml serum, respectively). The increase in pulse amplitude was immediate, but was less ($P < 0.05$) during the second 12 h, compared to the first 12 h, of naloxone infusion (0.52 ± 0.14 vs 0.98 ± 0.08 ng/ml serum). Oestradiol concentrations were higher ($P < 0.01$) during naloxone than during control infusion (5.63 ± 0.26 vs 4.13 ± 0.15 pg/ml serum). In ovariectomized ewes in the breeding season, LH pulse frequency was lower ($P < 0.01$) during a 24 h infusion of morphine (0.5 mg/kg/h) than during a 24 h infusion of vehicle (mean $\pm$ s.e.m.; 1.17 ± 0.08 vs 1.71 ± 0.06 pulses/h). We conclude that long-term infusion of naloxone results in a sustained increase in LH pulse frequency but only a transient elevation in pulse amplitude. No effects on FSH secretion were noted. LH secretion was sensitive to morphine in the absence of ovarian steroids, suggesting that ovarian steroids are not required for the presence of functional opioid receptors capable of modulating LH release.

Keywords: LH; FSH; ewe; morphine; naloxone

Introduction

Endogenous opioid peptides have been isolated from the sheep pituitary (Li *et al.*, 1965; Silman *et al.*, 1979; Leisti *et al.*, 1982) and hypothalamus (Ebling *et al.*, 1987) and specific receptors have been documented in various areas of the brain (Dunlap *et al.*, 1986). Endogenous opioids suppress LH secretion in the ewe (Malven *et al.*, 1984; Schillo *et al.*, 1985; Brooks *et al.*, 1986a, b, c; Gregg *et al.*, 1986; Currie & Rawlings, 1987; Trout & Malven, 1987; Yang *et al.*, 1988), as in other species (Bhanot & Wilkinson, 1983a, b). Work in mature rodents (Bhanot & Wilkinson, 1983b) and ewes (Currie & Rawlings, 1987) suggests that opioids may not modulate FSH secretion.

Inhibition of LH secretion by endogenous opioids in sheep may be mediated primarily at the hypothalamus (Cuttler *et al.*, 1985; Ebling & Lincoln, 1985; Schanbacher, 1985; Brooks *et al.*, 1986b; Malven, 1986; Currie & Rawlings, 1987), but direct pituitary effects have been shown in sheep (Matteri & Moberg, 1985), cattle (Chao *et al.*, 1986) and rat (Blank *et al.*, 1986; Cacicedo & Franco, 1986) pituitary cell cultures. A clear characterization of the effects of opioids on basal LH pulse amplitude and frequency may help to clarify the site of inhibition. The inhibitory influence of opioids on LH secretion may be present, but reduced, in the absence of ovarian steroids in the ewe (Schillo *et al.*, 1985). As in other species, opioids may mediate steroid negative feedback on basal LH secretion (Malven *et al.*, 1984; Brooks *et al.*, 1986a; Currie & Rawlings, 1987).

In most species, studies of the effect of opioids on LH secretion have involved short periods of sampling, and when antagonists such as naloxone were given, a nearly immediate pulse of LH was

seen (Matthews & Murdock, 1984; Gregg *et al.*, 1986; Malven, 1986; Currie & Rawlings, 1987). These factors make conclusions on the effects of endogenous opioids on basal LH pulse frequency and amplitude rather equivocal. In male rats (Miller *et al.*, 1986; Owens & Cicero, 1986) and rams (Ebling & Lincoln, 1985) a shift in the responsiveness of LH secretion to naloxone occurred when naloxone was given on subsequent days, and so long- and short-term effects may vary.

The purpose of this study was to use prolonged infusion of an opiate agonist, morphine, and an opiate antagonist, naloxone, to investigate the role of endogenous opioids in FSH secretion and to describe accurately the effect on basal LH secretion in the ewe. The latter might help indicate site of action and characterize short- and long-term effects. We also wished to confirm and describe the effect of morphine in the ovariectomized ewe. Infusion of morphine was performed in chronically ovariectomized ewes because fluctuations in the purported negative response of LH to morphine would be readily demonstrated in ewes secreting gonadotrophins at a nearly maximal rate in the absence of ovarian steroid feedback. Infusion of naloxone was performed in ewes in the mid-luteal phase of the oestrous cycle, as fluctuations in the purported positive response of LH to naloxone would be readily demonstrated in ewes secreting gonadotrophins at low levels in the presence of ovarian progesterone and oestradiol feedback.

Materials and Methods

Animals and experimental procedures. Six intact Suffolk ewes and 6 ovariectomized Columbia ewes were housed outdoors. Bilateral ovariectomy was performed at least 150 days before experimentation. Oestrus was synchronized in the intact ewes with intravaginal sponges containing 60 mg medroxyprogesterone acetate (Veramix, Tuco Products Co., Orangeville, Ontario, Canada) and vasectomized rams were used for detection of oestrus. Ewes were brought indoors 4 days before infusion, and catheterized (vinyl tubing, SV-70, i.d. 1.00 mm, o.d. 1.50 mm; Dural Plastics and Engineering, Dural, New South Wales, Australia) in both jugular veins 1 day before infusion. On Day 8 of the oestrous cycle, intact ewes were infused with vehicle (0.154 M-NaCl) for 24 h and on Day 9 with 0.5 mg naloxone/kg/h (E. I. DuPont de Nemours & Co., Inc., Garden City, New York, USA) in vehicle for 24 h. Ovariectomized ewes were infused with vehicle and with 0.5 mg morphine/kg/h (Ingram and Bell, Ltd, Don Mills, Ontario, Canada) in vehicle over 2 consecutive periods of 24 h. During infusion, ewes were restrained in crates, with food and water available. A peristaltic pump (Manostat, New York, New York, USA) connected to the left jugular catheters via silicone rubber tubing (i.d. 2.38 mm, o.d. 3.97 mm; Manostat) was used for the infusions. The dose of naloxone had been determined to elevate basal serum LH concentration in ewes during the early and mid-luteal and early follicular phases (Currie & Rawlings, 1987). The dose of morphine had been determined to decrease either LH pulse amplitude or frequency in ovariectomized ewes. Blood samples (3 ml) were collected at 12 min intervals from the right jugular catheter throughout infusion periods. Blood samples were allowed to clot for 1 h at 21°C, then 4 h at 4°C. Clots were removed, serum was centrifuged at 1500 g for 20 min, separated from remaining red cells and stored at -20°C.

Hormone assays. Serum concentrations of LH and FSH were determined, in all samples, by validated (Rawlings *et al.*, 1984) double-antibody radioimmunoassay and are expressed in terms of NIAMDD-oLH-24 and NIAMDD-oFSH-RP-1, respectively. Assays were unmodified other than that the anti-FSH serum was NIAMDD-anti-oFSH-1 raised in rabbits and used at a final dilution of 1:72 000. Sensitivities of the assays, defined as the concentrations of the lowest standards different ($P < 0.05$) from zero, were 0.06 ng LH/ml serum and 0.13 ng FSH/ml serum. Intra ($n = 10$) and inter-assay ($n = 20$) coefficients of variation (CVs) for LH were 3.28% and 4.73%, respectively, for a high reference serum (mean = 2.56 ng/ml) and 7.25% and 13.0%, respectively, for a low reference serum (mean = 0.39 ng/ml). Intra- ($n = 10$) and inter-assay ($n = 20$) CVs for FSH were 3.52% and 5.00%, respectively, for a high reference serum (mean = 8.06 ng/ml) and 12.3% and 13.1%, respectively, for a low reference serum (mean = 0.86 ng/ml).

Serum concentrations of oestradiol and progesterone were determined, in pooled hourly samples, using validated assays (Rawlings *et al.*, 1984). Sensitivities of the assays were 0.03 ng progesterone/ml serum and 1.0 pg oestradiol/ml serum. Intra- ($n = 4$) and inter-assay ($n = 12$) CVs, respectively, for the oestradiol reference sera were 13.5% and 17.0% (mean = 6.2 pg/ml), 11.2% and 17.8% (mean = 7.7 pg/ml), 10.8% and 17.0% (mean = 9.2 pg/ml) and 10.7% and 13.4% (mean = 16 pg/ml). The assay blank in the oestradiol assay was not different from zero. All samples analysed for progesterone were included in one assay with a CV ($n = 8$) of 9.59% for a low reference serum (mean = 1.03 ng/ml) and 8.46% for a high reference serum (mean = 2.52 ng/ml). The assay blank in the progesterone assay was not different from zero. Extraction efficiency was $86 \pm 1.2\%$ (mean $\pm$ s.e.m.) for oestradiol and 78% for progesterone.

LH pulses were detected as values above basal LH by Pulsar (Merriam & Wachter, 1982) using standard deviation criteria for identification of peaks within grouped points in scaled residual series selected by analysis of, approximately, 1100 h of control data obtained by bleeding intact and ovariectomized ewes at 12 min intervals. The interpolating function for the assay standard deviation was determined by analysis of the variation of standard curves

from 24 assays, each with a minimum of 7 standard doses. FSH was not released in a pulsatile manner as demonstrated by this method. Data were analysed by one-way analysis of variance.

Results

The effects of a 24 h infusion of naloxone on circulating gonadotrophin concentrations in the ewe, during the mid-luteal phase and of morphine in the ovariectomized ewe are summarized in Tables 1 and 2, respectively. The temporal patterns of LH concentrations in 1 ovariectomized ewe during control and morphine infusions and 1 intact ewe in the mid-luteal phase during control and naloxone infusions are illustrated in Figs 1(a) and (b). The temporal patterns of oestradiol and progesterone in intact ewes during control and naloxone infusions are illustrated in Figs 1(c) and (d).

Table 1. Serum LH concentration, LH pulse amplitude, LH pulse frequency and serum FSH concentration in ewes (N = 6) infused with vehicle, or with naloxone (0.5 mg/kg/h) in vehicle, for 24 h during the mid-luteal phase of the oestrous cycle

	LH conc. (ng/ml)	LH pulse amplitude (ng/ml)	LH pulse frequency (pulses/h)	FSH conc. (ng/ml)
Control	0.15 ± 0.01	0.55 ± 0.08	0.14 ± 0.01	0.99 ± 0.09
Naloxone	0.20 ± 0.02	0.84 ± 0.08*	0.25 ± 0.03**	1.04 ± 0.09

Values are mean ± s.e.m.

* $P < 0.05$, ** $P < 0.01$ compared with control value.

Table 2. Serum LH concentration, LH pulse amplitude, LH pulse frequency and serum FSH concentration in ewes (N = 6) infused with vehicle, or with morphine (0.5 mg/kg/h) in vehicle, for 24 h

	LH conc. (ng/ml)	LH pulse amplitude (ng/ml)	LH pulse frequency (pulses/h)	FSH conc. (ng/ml)
Control	3.16 ± 0.67	1.41 ± 0.34	1.71 ± 0.06	16.2 ± 0.69
Morphine	3.11 ± 0.36	1.63 ± 0.10	1.17 ± 0.08*	17.9 ± 0.40

Values are mean ± s.e.m.

* $P < 0.01$ compared with control value.

Neither morphine nor naloxone affected FSH concentrations. Infusion of morphine resulted in suppression ($P < 0.01$) of LH pulse frequency while mean serum LH concentration and LH pulse amplitude were not affected.

Both LH pulse amplitude ($P < 0.05$) and LH pulse frequency ($P < 0.01$) were increased by infusion of naloxone in intact ewes. The positive effect of naloxone on LH pulse amplitude was transient. LH pulse amplitude (mean ± s.e.m.) was greater ($P < 0.05$) during the first 12 h than during the second 12 h of naloxone infusion (0.98 ± 0.08 vs 0.52 ± 0.14 ng/ml serum) and LH pulse amplitude during the second 12 h of naloxone infusion was not different from the control values determined over the first or second 12 h, or over the entirety, of the control

Fig. 1. The effects of morphine on LH concentrations in one ovariectomized ewe during the breeding season (a), the effects of naloxone on serum LH concentrations in one intact ewe during the mid-luteal phase (b) and the effects of naloxone on the temporal patterns (mean $\pm$ s.e.m.) of oestradiol (c) and progesterone (d) in serum of intact ewes during the mid-luteal phase. Vehicle (control) was infused for 24 h, followed by drug for 24 h. Blood samples were taken at 12-min intervals.

infusion. The elevation of LH pulse frequency was maintained throughout the infusion of naloxone. During infusion of naloxone, circulating oestradiol concentrations were elevated ($P < 0.01$) (4.13 ± 0.15 vs 5.63 ± 0.26 pg/ml serum, control vs naloxone), while the apparent increase in progesterone (control: 2.52 ± 0.88 ; naloxone: 2.83 ± 0.16 ng/ml serum) was not significant. Regression of circulating oestradiol on LH pulse amplitude, after division of data accumulated during naloxone infusion into 4 h blocks, gave a positive correlation (0.82), with oestradiol increasing shortly after the naloxone induced increase in LH pulse amplitude, early

in naloxone infusion, and decreasing as LH pulse amplitude decreased during the remainder of naloxone infusion.

Discussion

Endogenous opioid peptides may tonically inhibit LH release in the ewe. The enhancement of LH pulse frequency by naloxone was maintained throughout the 24 h infusion period in this study. However, a loss of naloxone enhancement of LH pulse frequency, with prolonged exposure to naloxone, has been reported for intact and pinealectomized rams (Ebling & Lincoln, 1985). A prolonged period of observation, as in this study, would allow a more accurate estimate of pulse frequency. In addition, in this and previous studies, it has been observed that naloxone administration is followed nearly immediately by an LH pulse (Matthews & Murdock, 1984; Gregg *et al.*, 1986; Malven, 1986; Currie & Rawlings, 1987). This phenomenon may cause an overestimation of LH pulse frequency, especially in studies using a short period of observation. Such bias would have been reduced with the increased sampling duration used in this study.

In this study, the response of LH pulse amplitude to infusion of naloxone was transient. We previously reported an increase in LH pulse amplitude during infusion of naloxone in the ewe during the early and mid-luteal and early follicular phases, but not during the late luteal phase of the oestrous cycle (Currie & Rawlings, 1987), when LH pulse amplitude was high but more variable. It has been suggested that changes in LH pulse amplitude after naloxone administration may be mediated at the level of the pituitary, involving an increase in responsiveness to successive GnRH pulses, as a function of an increased GnRH pulse frequency (Ebling & Lincoln, 1985). This may not explain the response pattern observed in this study, in which LH pulse amplitude increased immediately upon infusion of naloxone, but gradually decreased, despite a constantly elevated LH pulse frequency. The enhanced LH pulse amplitude may have been due to recruitment of additional GnRH-releasing neurones during secretion of each GnRH pulse or due to loss of opioid tone over LH release at the pituitary, effects which may have been lost with prolonged exposure to naloxone.

Several hypotheses for the loss of responsiveness of LH pulse amplitude after prolonged naloxone exposure in the male rat have been reviewed (Miller *et al.*, 1986). In comparison with the prolonged release of LH, and presumably GnRH, during the preovulatory surge (Jeffcoat *et al.*, 1984) in the ewe, the magnitude of the enhancement of LH release by naloxone observed in the present study is small. It is unlikely, therefore, that the decrease in responsiveness of LH pulse amplitude to prolonged infusion of naloxone was due to depletion of LH or GnRH stores. Nor would it seem likely that this effect was due to hepatic enzyme induction over the duration of 12 h. A tolerance-like character of a similar decrease in LH responsiveness to naloxone has been demonstrated in the rat (Owens & Cicero, 1986). Auto-regulation of hypothalamic opioid receptors is evidenced by an increased release of opioid peptides from hypothalamic neurons treated with naloxone (Nikolarakis *et al.*, 1986), suggesting the possibility that prolonged treatment with naloxone may have resulted in an increased release of opioids which gradually suppressed LH pulse amplitude. The possibility of negative co-operativity may also exist, since numerous receptor types have been identified within the opioid receptor population. It has been suggested that the transient response of LH to naloxone in the male rat may be due to enhanced steroid feedback, with an increase in testosterone occurring within the first 2 h of naloxone administration (Miller *et al.*, 1986). The results of the present experiment indicate that enhanced steroidogenesis during early naloxone exposure resulted in an increased level of gonadal steroid feedback by oestradiol, which may have overshadowed LH responsiveness to naloxone during prolonged naloxone exposure. The positive correlation between circulating oestradiol and LH pulse amplitude during naloxone infusion in this study suggests that an increased LH pulse amplitude serves as a stimulus for increased

secretion of oestradiol, and that the resulting higher level of oestradiol then suppressed LH pulse amplitude, lessening the stimulus for oestradiol secretion. Thus, the two hormones rise and fall in near synchrony.

In this study, in the chronically ovariectomized ewe, stimulation of opioid receptors by morphine suppressed LH pulse frequency while LH pulse amplitude was not significantly affected. The lack of an accompanying decrease in mean LH concentration may have been due to the apparent, but not significant, rise in LH pulse amplitude, possibly a reflection of the inverse relationship between GnRH pulse frequency and LH pulse amplitude which occurs in the ewe (Kaynard & Karsch, 1983). This suppression of LH release by morphine in the chronically ovariectomized ewe suggests that opioid receptors are present and functional in the absence of gonadal steroids. This finding differs from that reported for the rat (Bhanot & Wilkinson, 1983b), but is similar to observations of ewes ovariectomized for at least 90 days (Schillo *et al.*, 1985) and in wethers 6 months after orchidectomy (Schanbacher, 1985).

In the present study, neither morphine infusion of the ovariectomized ewes nor naloxone infusion of the intact ewes during the mid-luteal phase affected mean serum FSH concentration. We previously reported that naloxone infusion, at the dose used in this study, did not affect mean serum FSH concentration during the early, mid- or late luteal or early follicular phases of the oestrous cycle (Currie & Rawlings, 1987). The lack of effects of opioid receptor stimulation or blockade on FSH may suggest the presence of an alternative FSH-releasing factor or an insensitivity of distinct gonadotrophs secreting FSH (Naor & Childs, 1986) to short-term fluctuations in GnRH release. Female human opiate addicts, however, fail to exhibit normal cyclic fluctuations in FSH concentrations (Santen *et al.*, 1975), although this may be secondary to alteration in gonadal steroid feedback due to insufficient LH support of ovarian structures. Opiate agonists and antagonists have been reported to suppress and enhance, respectively, FSH concentrations in intact and ovariectomized immature (Bhanot & Wilkinson, 1983a), but not mature (Bhanot & Wilkinson, 1983b), rats. Naloxone (Grossman *et al.*, 1986) increased FSH in short-term studies in men, while opiate agonists (Pende *et al.*, 1986) were without effect.

Endogenous opioids may tonically inhibit LH release in the ewe. Evidence gained in this study indirectly implicates opioid activity at the level of the neural GnRH oscillator, but does not eliminate the possibility of activity at the level of the pituitary. Prolonged opioid receptor blockade enhanced both LH pulse frequency and LH pulse amplitude, but the responsiveness of LH pulse frequency to naloxone was prolonged beyond the responsiveness of LH pulse amplitude. The responsiveness of LH pulse amplitude to opioid receptor blockade was immediate and may implicate recruitment of additional GnRH neurones during secretion of each GnRH pulse, or blockade of opioid suppression of gonadotrophin secretion at the pituitary, rather than enhanced self priming of pituitary responsiveness to successive GnRH pulses by an increased GnRH pulse frequency. The loss of responsiveness of LH pulse amplitude observed during the second half of the naloxone infusion may have been a pituitary phenomenon mediated by enhanced LH support of ovarian steroidogenesis which enhanced steroid feedback, perhaps by oestradiol, at the pituitary. In the absence of ovarian steroid feedback in the ovariectomized ewe, stimulation of the opioid receptor by morphine only affected LH pulse frequency. There was no effect of morphine infusion on LH pulse amplitude. This paradoxical observation would suggest that endogenous opioids have no effect on LH pulse amplitude. However, this observation may reflect the loss of some component of pituitary or hypothalamic responsiveness to modulation by opioids in the chronically ovariectomized ewe.

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