

# Inhibition of gonadotrophin release in mares during the luteal phase of the oestrous cycle by endogenous opioids

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Effects of the opioid antagonist naloxone on concentrations of LH and FSH in plasma were measured in mares during different stages of the oestrous cycle. During the follicular phase of the cycle, naloxone (300 mg i.v.) had no discernible effects on basal concentrations of LH and FSH. A significant increase in plasma LH ( $P < 0.01$ ) and FSH ( $P < 0.05$ ) concentrations was observed after naloxone in mares during the luteal phase. This response was not different between suckled and non-suckled mares. The gonadotrophin-releasing hormone analogue buserelin (0.02 mg i.v.) caused a significant ( $P < 0.05$ ) LH and FSH release irrespective of the stage of the oestrous cycle and a previous naloxone treatment. The results of this study indicate that endogenous opioid peptides are involved in the inhibition of LH and FSH release during the luteal phase of the oestrous cycle in mares and may partially mediate the suppressive influence of progesterone on gonadotrophin secretion. The opioid-mediated suppression of LH and FSH release does not seem to be affected by suckling.

## Introduction

Endogenous opioid peptides appear to be involved in the regulation of gonadotrophin release. It has been suggested that opioids inhibit the release of LH in cyclic women (Quigley and Yen, 1980; Blankstein *et al.*, 1981) and other primates (Orstead *et al.*, 1987), rats (Petraglia *et al.*, 1986; Allen *et al.*, 1988), sheep (Currie and Rawlings, 1987; Malven and Hudgens, 1987), pigs (Barb *et al.*, 1986) and cattle (Short *et al.*, 1987) on the basis of opioid antagonists increasing concentrations of LH in blood. However, there are differences between species. The opioidergic modulation of LH secretion is influenced by gonadal steroids and changes with the stage of the oestrous cycle. Whereas in all species investigated opioids inhibit LH release during either the luteal or parts of the follicular phase, an opioid influence on the secretion of FSH is less clear, but could be demonstrated in humans (Blankstein *et al.*, 1981) and rats (Bedran de Castro *et al.*, 1986). In addition to certain stages of the oestrous cycle, opioids inhibit LH secretion during *postpartum* anoestrus. The opioid mediated suppression of LH release depends on suckling in cows (Whisnant *et al.*, 1986) and sows (Armstrong *et al.*, 1988) and is independent of the suckling stimulus in sheep (Malven and Hudgens, 1987).

The oestrous cycles of mares and other domestic animals differ in several aspects. In contrast to pigs and cattle, horses have a seasonal breeding activity and a prolonged transitional period between winter anoestrus and cyclic ovarian activity. A gradual preovulatory increase in LH secretion begins about 4 days before ovulation (Miller *et al.*, 1980) and oestrus lasts for 2

to 11 days with a mean of 7 days (Adams and Bosu, 1988). The duration of oestrus in mares is therefore longer and much more variable than in rats, domestic ruminants and pigs.

In this study we investigated the influence of the opioid antagonist naloxone on LH and FSH release in mares at different stages of the oestrous cycle and whether these effects are different in suckled and non-suckled mares. If endogenous opioids participate in the regulation of LH and FSH secretion, naloxone should lead to an increased release of gonadotrophins.

## Materials and Methods

### Animals

Brood mares ( $n = 25$ ) of the Hanoverian breed, aged between 3 and 12 years ( $7.3 \pm 2.7$  years,  $\pm$ SD), were used. The weight of the mares was  $590 \pm 21$  kg ( $\pm$ SD). Animals belonged to the stud farm of the state of Sachsen-Anhalt (Radegast). Horses were housed in loose boxes and were fed oats and hay three times daily, water was freely available.

Mares were teased for oestrous behaviour with a stallion three times a week. Ovaries were examined for follicles and corpora lutea by rectal palpation and ultrasound scanning with a 5 MHz linear scanner (Aloka; Eickemeyer, Tuttlingen).

Mares were considered to be in the follicular phase when they showed oestrous behaviour (immobile stance, clitoral winking, tail raise) during teasing, had a follicle with a diameter of  $>30$  mm and a plasma progesterone concentration of  $<0.8$  ng ml<sup>-1</sup>. Mares were considered to be in the luteal phase when they had not shown oestrous behaviour for 6 to 12 days,

a corpus luteum was detectable by ultrasound examination and the progesterone concentration in plasma was  $>0.8 \text{ ng ml}^{-1}$  (see Table 1). Mares at oestrus and at the luteal phase were randomly assigned to the experimental or control groups.

### Experimental protocol

A total of 41 treatments were performed between April and June 1992. Sixteen mares were used twice, that is during the follicular and luteal phase.

An indwelling catheter was placed in the left jugular vein 15 min before taking the first blood sample. During sampling mares were left in their stables. In suckled mares, the foals were present during the experiment.

Blood samples for determination of LH and FSH were taken at 15 min intervals for 4 h. After 60 min of sampling, naloxone-HCl (300 mg per mare; Sigma, Deisenhofen) or 10 ml of saline were injected via the catheter into the jugular vein. Naloxone was freshly dissolved in 10 ml of saline and sterile filtered. Two hours after injections of naloxone or saline, 20  $\mu\text{g}$  of the GnRH analogue buserelin (Receptal; Hoechst, Unterschleißheim) was administered to all animals and blood samples were collected for another 60 min (i.e. until 180 min after naloxone or saline). Plasma progesterone and oestradiol concentrations were measured in the first sample taken on each day.

Blood was collected into polystyrol tubes containing 25 mg EDTA. Samples were centrifuged immediately for 20 min at 1000 g; plasma was frozen at  $-20^\circ\text{C}$  and was assayed within 3 weeks.

### Hormone analysis

Concentrations of LH in plasma were measured by radioimmunoassay as described by Pantke *et al.* (1991) with an antibody raised in rabbits against equine LH (UCB, Braine l'Alleu). The antiserum was diluted in  $0.14 \text{ mol NaCl l}^{-1}$ ,  $0.01 \text{ mol NaH}_2\text{PO}_4 \text{ l}^{-1}$  (PBS; pH 7.2), containing 1% (v/v) normal rabbit serum. Crossreactivity of the antibody with FSH was less than 0.1%. Plasma samples (50  $\mu\text{l}$ ) or standards (100  $\mu\text{l}$ ; UCB) were incubated with 100  $\mu\text{l}$  antiserum in a total of 300  $\mu\text{l}$  PBS containing 1% (w/v) BSA for 24 h at room temperature. The standard preparation used has also been validated in an LH bioassay *in vitro* (Pantke *et al.*, 1991). Final dilution of the antiserum was 1:85 000. For determination of nonspecific binding, normal rabbit serum was added instead of antiserum.  $^{125}\text{I}$ -labelled equine LH, prepared with the chloramine-T method (Greenwood *et al.*, 1963) was used as tracer at a dilution of 20 000 c.p.m. in 100  $\mu\text{l}$  PBS containing 1% BSA. After addition of the tracer, incubation was continued for another 24 h at  $4^\circ\text{C}$ . Bound ligands were precipitated by adding 200  $\mu\text{l}$  of sheep anti-rabbit IgG at a dilution of 1:20 in PBS containing 6% (w/v) polyethylene glycol (molecular weight 20 000). Incubation was continued for 1 h at room temperature and 30 min at  $4^\circ\text{C}$ . Bound and free ligands were separated by centrifugation at  $4^\circ\text{C}$  for 20 min and 2000 g. The supernatant was discarded and radioactivity in the pellet measured in a gamma-counter (Multigamma 1261; Pharmacia, Freiburg). The minimal detectable concentration of this assay was  $0.5 \text{ ng ml}^{-1}$ , zero binding was 25% and intra- and interassay coefficients of variation were, respectively, 5.8% and 18.1%.

FSH was measured by radioimmunoassay with an antiserum raised in rabbits against equine FSH (RB 530: H. Papkoff, University of California) and an equine FSH standard preparation (E276B: H. Papkoff). The antiserum was used at a final dilution of 1:80 000 in  $0.14 \text{ mol NaCl l}^{-1}$ ,  $0.001 \text{ mol NaH}_2\text{PO}_4 \text{ l}^{-1}$  (pH 7.2) containing 1% (v/v) normal rabbit serum. Cross-reactivity of the antibody with LH was 2.8%.  $^{125}\text{I}$ -labelled FSH, prepared by the chloramine-T method (Greenwood *et al.*, 1963), was used as tracer at a dilution of 20 000 c.p.m. in 100  $\mu\text{l}$ . The assay procedure for FSH was similar to that described for LH with the modification that 25  $\mu\text{l}$  instead of 50  $\mu\text{l}$  plasma was used. The minimal detectable concentration of the radioimmunoassay for FSH was  $0.15 \text{ ng ml}^{-1}$ ; zero binding was 20% and intra- and interassay coefficients of variation were 7.9% and 8.4%, respectively.

Progesterone was determined by radioimmunoassay after extraction from plasma (0.1 ml) with *n*-hexane (1 ml). The antibody crossreacted 51.5% with 11-dehydropregesterone, 34.0% with  $17\alpha$ -hydroxyprogesterone, 2.2% with  $16\alpha$ -hydroxyprogesterone and less than 0.9% with  $20\alpha$ -dihydropregesterone, pregnenolone,  $17\alpha$ -hydroxypregnenolone, corticosterone, cortisol, aldosterone, testosterone, androstendione, oestradiol,  $17\alpha$ -oestradiol, oestrone and oestriol. Dried down sample extracts were resuspended in 100  $\mu\text{l}$   $0.01 \text{ mol NaH}_2\text{PO}_4 \text{ l}^{-1}$ , containing 0.01 mol EDTA  $\text{l}^{-1}$  and 0.1% (w/v) gelatine (pH 7.2). [ $1,2,6,7\text{-}^3\text{H}$ ]progesterone (Amersham Buchler, Braunschweig) at a dilution of 7000 c.p.m. in 100  $\mu\text{l}$  and antiserum at a dilution of 1:50 000 in 100  $\mu\text{l}$  were added. After incubation at  $4^\circ\text{C}$  for 18 h, free and bound ligands were separated by dextran-coated charcoal. The supernatant was mixed with 1 ml liquid scintillation fluid (Ready Organic, Beckman Instruments, Munich) and radioactivity was measured in a  $\beta$ -counter. The minimal detectable concentration was  $50 \text{ pg ml}^{-1}$ ; zero binding was 42% and intra- and interassay coefficients of variation were 4.5% and 9.7%, respectively.

Oestradiol was extracted from plasma (0.3 ml) with ethylacetate (3 ml) and determined by radioimmunoassay. The antiserum (GDN 244: G. Niswender, Colorado State University) was used at a final dilution of 1:250 000. Crossreactivity was 52% with 4-hydroxyoestradiol, 22% with 2-hydroxyoestradiol, 2.8% with 4-hydroxyoestrone, 1.8% with oestrone, 1.0% with oestriol, 0.9% with  $17\alpha$ -oestradiol, 0.1% with 2-hydroxyoestrone, 0.07% with  $5\alpha$ -dihydrotestosterone and less than 0.02% with progesterone,  $17\alpha$ -hydroxyprogesterone, pregnenolone, tamoxifen, 4-hydroxytamoxifen, dehydroepiandrosterone, androstendione, testosterone and cortisol. [ $2,4,6,7\text{-}^3\text{H}$ ]oestradiol (Amersham Buchler) was used as tracer at a dilution of 8000 c.p.m. in 100  $\mu\text{l}$ . The radioimmunoassay was performed as described for progesterone with the modification that  $0.01 \text{ mol NaH}_2\text{PO}_4 \text{ l}^{-1}$ ,  $0.01 \text{ mol EDTA l}^{-1}$ , 0.1% lysozyme (w/v; pH 7.2) was used as assay buffer. The minimal detectable concentration was  $2 \text{ pg ml}^{-1}$  and zero binding was 44%. Intra- and interassay coefficients of variation were 9.5% and 11.8%, respectively.

### Statistical analysis

Comparisons of LH and FSH values between groups were made by analysis of variance for repeated measures. This procedure takes into account the fact that sequential values were

**Table 1.** Progesterone and oestradiol concentrations in plasma of mares during the follicular and luteal phases of the oestrous cycle

Phase of oestrous cycle	Group	Number of animals (suckled/non-suckled)	Progesterone (ng ml <sup>-1</sup> )	Oestradiol (pg ml <sup>-1</sup> )
Follicular phase	Naloxone	10 (7/3)	0.21 ± 0.15 <sup>a</sup>	25.8 ± 11.7 <sup>c</sup>
	Saline	8 (5/3)	0.23 ± 0.23 <sup>a</sup>	30.9 ± 9.3 <sup>c</sup>
Luteal phase	Naloxone	12 (6/6)	3.35 ± 1.98 <sup>b</sup>	14.4 ± 8.9 <sup>d</sup>
	Saline	11 (4/7)	3.17 ± 1.92 <sup>b</sup>	16.7 ± 7.0 <sup>d</sup>

Values are means ± SEM.

a, b and c, d: Values in the same column with different letters are significantly different (a, b:  $P < 0.001$ ; c, d:  $P < 0.05$ ; one-way analysis of variance).

analysed. The analysis was made separately for the periods before naloxone or saline injection, between naloxone or saline and buserelin application and after buserelin treatment. In addition, LH and FSH concentrations before and after application of buserelin were analysed by paired samples *t* test, comparing the mean of the four values before buserelin injections with the mean of the four values after buserelin. Progesterone and oestradiol concentrations between groups were compared by one-way analysis of variance. All statistical comparisons were made with the SPCC/PC+ statistics package (Norusis, 1988). Values given are means ± standard error of mean (SEM).

## Results

### Steroid hormones

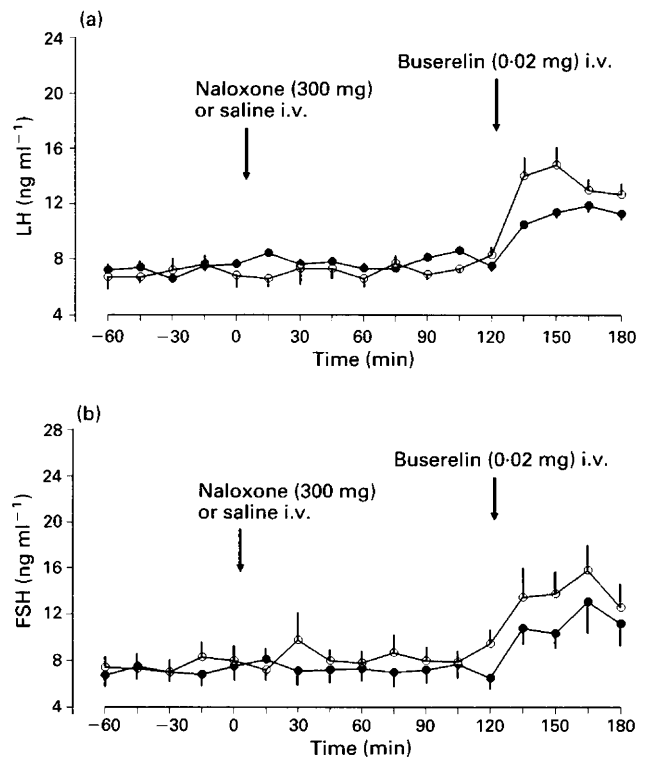
There were significant differences in plasma progesterone ( $P < 0.001$ ) and oestradiol ( $P < 0.05$ ) concentrations between mares in the luteal and follicular phase of the cycle (see Table 1). As can be expected from the experimental design, progesterone values were higher and oestradiol values lower during the luteal phase than during the follicular phase.

### LH and FSH

Concentrations of LH and FSH did not differ significantly between mares that were injected with naloxone and those that received saline during the follicular phase of the oestrous cycle (Fig. 1). Fifteen minutes before injection of naloxone LH and FSH concentrations were  $7.5 \pm 0.7$  and  $6.8 \pm 0.9$  ng ml<sup>-1</sup>, respectively. Thirty minutes after naloxone injection plasma LH concentrations were  $7.6 \pm 0.6$  ng ml<sup>-1</sup> and FSH concentrations  $7.1 \pm 1.1$  ng ml<sup>-1</sup>.

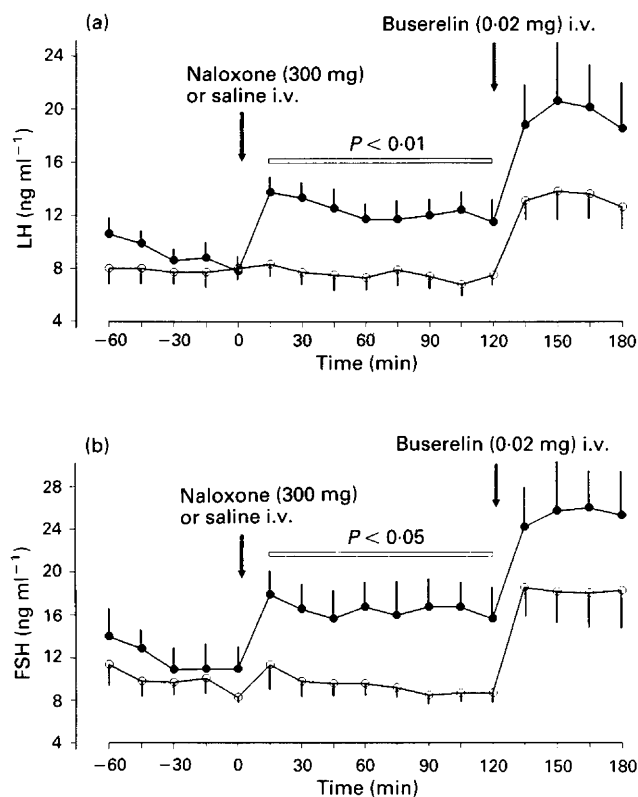
Application of 20 µg buserelin 2 h after naloxone resulted in a significant increase ( $P < 0.05$ ) in plasma LH and FSH concentrations in mares of both the control and treatment groups. Fifteen minutes after buserelin plasma LH and FSH concentrations were 10.5 and 10.8 ng ml<sup>-1</sup>, respectively, for mares that received naloxone and  $14.0 \pm 2.6$  and  $13.5 \pm 2.3$  ng ml<sup>-1</sup>, respectively, for mares that received saline.

During the luteal phase of the cycle, the application of 300 mg of naloxone resulted in a significant increase in LH ( $P < 0.01$



**Fig. 1.** Concentrations of (a) LH and (b) FSH in plasma of mares during the follicular phase of the oestrous cycle before and after injection of naloxone (●;  $n = 10$ ) or saline (○;  $n = 8$ ) and buserelin; values are means ± SEM. Analysis of variance for repeated measures showed that differences between groups were not statistically significant. Paired samples *t* test showed that LH and FSH values before and after injection of buserelin were significantly different for mares in both groups ( $P < 0.05$ ).

versus control group,  $DF$  1,21,  $F = 9.03$ ) and FSH ( $P < 0.05$  versus control group,  $DF$  1,21,  $F = 6.49$ ) concentrations in plasma (Fig. 2). Fifteen minutes before naloxone, plasma LH and FSH concentrations were  $8.8 \pm 1.0$  and  $11.0 \pm 2.2$  ng ml<sup>-1</sup>, respectively. Fifteen minutes after naloxone, concentrations of LH and FSH were  $13.7 \pm 4.1$  and  $17.9 \pm 8.1$  ng ml<sup>-1</sup>, respectively. Concentrations of both gonadotrophins remained high for 2 h after naloxone treatment, that is until the administration



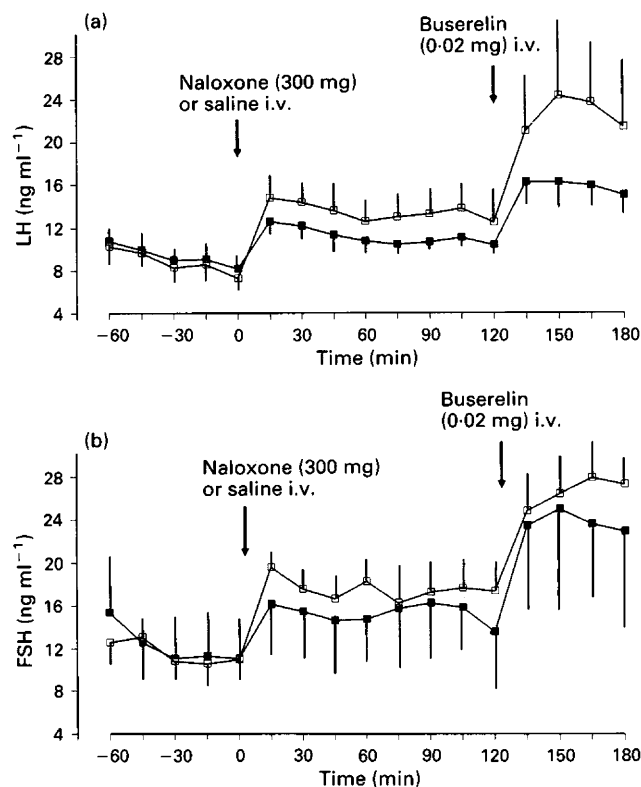
**Fig. 2.** Concentrations of (a) LH and (b) FSH in plasma of mares during the luteal phase of the oestrous cycle before and after injection of naloxone (●;  $n = 12$ ) or saline (○;  $n = 11$ ) and buserelin; values are means  $\pm$  SEM. Analysis of variance for repeated measures showed that values for groups are significantly different from each other for the period after naloxone or saline but before buserelin injections (LH  $P < 0.01$ ; FSH  $P < 0.05$ ). Paired samples  $t$  test showed that LH and FSH values before and after injection of buserelin were significantly different for mares in both groups ( $P < 0.05$ ).

of buserelin. Buserelin caused a further significant increase in plasma LH and FSH concentrations ( $P < 0.05$ ) in mares that had received naloxone and in mares that had received saline. Plasma concentrations of LH and FSH 15 min after injection of buserelin were  $18.8 \pm 2.9$  and  $24.3 \pm 3.7$  ng ml<sup>-1</sup>, respectively, in horses of the naloxone group. Corresponding values for mares that had received saline were  $13.1 \pm 1.6$  and  $18.6 \pm 2.7$  ng ml<sup>-1</sup>, respectively.

In non-sucked, luteal phase mares, the increase in gonadotrophin values tended to be higher than in sucked, luteal phase mares, but differences were not statistically significant (Fig. 3).

## Discussion

In this study we measured concentrations of LH and FSH in plasma of mares at different stages of the oestrous cycle before and after application of the opiate antagonist naloxone. Naloxone led to an increase in circulating LH and FSH concentrations in mares in the luteal phase but not in the follicular phase. These observations suggest that opioid peptides are



**Fig. 3.** Concentrations of (a) LH and (b) FSH in plasma of sucked (■;  $n = 6$ ) and non-sucked mares (□;  $n = 6$ ) during the luteal phase of the oestrous cycle before and after injection of naloxone and buserelin; values are means  $\pm$  SEM. Analysis of variance for repeated measures showed that differences between groups were not statistically significant. Paired samples  $t$  test showed that LH and FSH values before and after injection of buserelin were significantly different for mares in both groups ( $P < 0.05$ ).

involved in the regulation of gonadotrophin secretion during the oestrous cycle in horses.

An inhibitory action of opioid systems on LH release during the luteal phase of the cycle is in agreement with reports from humans (Quigley and Yen, 1980; Blankstein *et al.*, 1981), monkeys (Orstead *et al.*, 1987), rats (Petraglia *et al.*, 1986; Allen *et al.*, 1988), sheep (Currie and Rawlings, 1987; Malven and Hudgens, 1987) and pigs (Barb *et al.*, 1986). Inhibition of LH secretion by opioids was also demonstrated during parts of the follicular phase in humans (Quigley and Yen, 1980; Blankstein *et al.*, 1981) and rats (Petraglia *et al.*, 1986; Allen *et al.*, 1988). The opioid influence on LH release depends on gonadal steroids, especially progesterone. In ovariectomized women (Shoupe *et al.*, 1985) or sheep (Brooks *et al.*, 1986; Trout and Malven, 1987), naloxone influences LH release only after replacement of progesterone alone or with oestrogens. In species with a relatively short follicular phase, such as rats or sheep, oestrogens may act synergistically with residual progesterone from the corpus luteum in stimulating endogenous opioid systems. In mares, the period of high circulating oestrogen concentrations towards the end of oestrus occurs at least a week after luteolysis (Adams and Bosu, 1988). An oestrogenic influence alone does not seem to activate opioid systems involved in the regulation of gonadotrophin release.

In mares in the luteal phase, naloxone also induced a significant release of FSH. Changes in plasma FSH and LH concentrations occurred in parallel. A comparable effect of opioids on both gonadotrophins in the female has not been found in other species. In humans and rats, FSH release seems to be under some opioidergic control but the effect of opioid antagonists on FSH was less than on LH release. In sheep (Currie and Rawlings, 1987; Horton *et al.*, 1987) and rabbits (Younglai *et al.*, 1988), naloxone did not change plasma FSH concentrations. Opioids inhibit GnRH release from the hypothalamus (Kalra, 1981; Malven *et al.*, 1990). FSH should be affected by opioids in as much as it is under the control of GnRH. The different reaction of LH and FSH to opioid antagonists has been interpreted as evidence for factors in addition to GnRH that regulate LH and FSH release (Orstead and Spies, 1987; Currie and Rawlings, 1987). In mares, there is a strong coincidence between pulses of GnRH, LH and FSH (Alexander and Irvine, 1987). This is in agreement with a parallel reaction of LH and FSH to opioid receptor blockade in this study. From our results it can be concluded that in mares the negative feedback of progesterone or progesterone and oestrogens on gonadotrophin secretion is mediated at least in part by brain opioid systems. The decrease in circulating progesterone concentrations after luteolysis terminates the opioid inhibition of gonadotrophin release. The subsequent increased gonadotrophin secretion favours follicular development and finally ovulation. It might be argued that the lack of a reaction to naloxone in follicular phase mares could be due to insufficient pituitary gonadotrophin content. However, all mares reacted with a significant LH and FSH release after injection of a GnRH agonist. This result demonstrated that pituitary gonadotrophin content was not a limiting factor. It is also possible that a naloxone-induced LH and FSH release during the follicular phase was not of sufficient magnitude to be detectable in peripheral plasma.

A differential reaction to naloxone was not found in suckled and non-suckled mares. In pigs and beef cattle lactation is accompanied by a period of anoestrus. It has been suggested that endogenous opioids are released in response to the suckling stimulus and subsequently inhibit gonadotrophin secretion. Suckled sows and cows show an increased LH release after naloxone administration. This effect is no longer present after weaning (Whisnant *et al.*, 1986; Armstrong *et al.*, 1988). In sheep, however, naloxone increased plasma LH concentrations in weaned as well as in suckled ewes (Malven and Hudgens, 1987). In the study reported here, in suckled and non-suckled luteal phase mares LH and FSH concentrations increased after naloxone application. The increase tended to be even higher in the non-suckled mares but differences between suckled and non-suckled mares were not statistically significant. Our findings are therefore in agreement with results from sheep. In suckled cattle and sows, lactational anoestrus is characterized by the absence of cyclic ovarian activity (Cox and Britt, 1982; Walters *et al.*, 1982). In contrast, in the suckled mare a fertile oestrous cycle is present and suckling does not have a major effect on the opioid inhibition of gonadotrophin release.

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