Opioidergic regulation of gonadotrophin secretion in the early prepubertal bull calf

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The control of gonadotrophin secretion during the early developmental period in the bull is poorly understood; opioidergic regulation may be involved. The opioid antagonist naloxone was administered to bull calves (i.v. $n = 5$; 1 mg kg^{-1} body weight h^{-1}) for 12 h at 4, 12, 18, 24 and 32 weeks of age. Control animals ($n = 5$) received sterile saline solution. During the treatment period, 4 ml blood samples were collected every 12 min. The calves were also weighed and blood samples collected every two weeks from 2 to 48 weeks of age. Scrotal circumference was measured from 20 to 48 weeks of age to determine age at puberty. Serum samples were assayed for concentrations of LH, FSH, testosterone and oestradiol. On the basis of blood samples taken every two weeks, serum concentrations of LH increased to a peak at six weeks of age and then decreased; serum concentrations of FSH reached a peak at 20 weeks of age. Serum concentrations of testosterone and oestradiol increased with scrotal circumference from 20 to 48 weeks of age. On the basis of blood samples taken every 12 min for 12 h, we concluded that the early rise in serum LH concentrations was due to an increase in LH pulse frequency. LH pulse amplitude decreased from 4 to 18 weeks of age and FSH pulse amplitude from 4 to 32 weeks of age. Naloxone treatment increased LH pulse frequency at 4 and 24 weeks of age but not at 12, 18 or 32 weeks of age. LH pulse amplitude was depressed and mean serum concentrations of LH were increased by naloxone at 4 weeks of age. At 12 weeks of age, LH pulse amplitude and mean and basal serum LH concentrations were increased by naloxone. At 18 weeks, only mean concentrations of LH were increased by naloxone. The only effect of naloxone on FSH secretion was to increase FSH pulse amplitude at 24 weeks of age. We conclude that there is opioidergic inhibition of LH secretion in the young bull calf, but that between 12 and 18 weeks of age a decrease in opioidergic inhibition on LH pulse frequency contributes to the overall increase in mean circulating concentrations of LH at this time.

Introduction

Puberty has been defined as the phase of bodily development during which the gonads secrete hormones in amounts that cause accelerated growth of the genital organs and the appearance of secondary sexual characteristics (Donovan and van der Werff ten Bosch, 1965). Puberty has been considered to be the discrete developmental age when reproduction is possible (Killian and Amann, 1972; Barber and Almquist, 1975). Hence, the period before puberty is a period of sexual maturation in which gonadal development, bodily development and endocrine development take place. In the heifer, puberty is simply first ovulation; in the bull it is defined as the age at which an ejaculate contains 50×10^6 spermatozoa with a minimum motility of 10% (Wolf *et al.*, 1965). Regardless of breed, body weight and age, puberty has been correlated with a scrotal circumference of 27.9 ± 0.2 cm in bulls (Lunstra *et al.*, 1978).

The prepubertal pattern of circulating gonadotrophins in the bull calf has been established. In the immediate postnatal period, circulating concentrations of LH are low, and this gives way to an early transient rise in LH from about 10 until 20 weeks of age (Rawlings *et al.*, 1978; McCarthy *et al.*, 1979; Amann *et al.*, 1986). At about 24 weeks of age, LH concentrations start to increase to puberty (Rawlings *et al.*, 1972; Rawlings *et al.*, 1978; Schams *et al.*, 1981). Circulating concentrations of FSH show a similar pattern, except the early rise appears to last longer and the increase to puberty occurs earlier (Schams *et al.*, 1981). Serum testosterone concentrations are initially very low but start to increase at about 15–20 weeks of age and rise to puberty and on into early adulthood (Rawlings *et al.*, 1972; Secchiari *et al.*, 1976; Lunstra *et al.*, 1978; Rawlings *et al.*, 1978; Rawlings and Cook, 1986). In the young bull calf, when testosterone secretion is low, serum concentrations of androstenedione and some 5α -reduced androgens are high (Rawlings *et al.*, 1972; Abdel Malak Bedair and Thibier, 1979; Rawlings and Cook, 1986). Significant quantities of serum oestradiol and oestradiol receptors in the hypothalamus and median eminence

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have been measured in very young bull calves, with an indication that circulating oestradiol concentrations reach a maximum at six weeks of age and are low thereafter (Amann *et al.*, 1986).

The exact mechanisms controlling sexual maturation in bulls are not understood. It would appear that control is at the level of the hypothalamus, since the pituitary in very young bulls is responsive to exogenous GnRH when gonadotrophin secretion is otherwise low (Lacroix and Pelletier, 1979; Schams *et al.*, 1981; Rodriguez and Wise, 1989) and the testes produce pulses of testosterone in response to endogenous pulses of LH (Karg *et al.*, 1976; Schams *et al.*, 1978).

The involvement of opioid peptides in regulating the onset of puberty in various species has been considered (Wilkinson and Landymore, 1989; Barb *et al.*, 1991). In rats, precocious puberty has been induced in females by the early administration of the opioid receptor antagonist naloxone (Sirinathsinghji *et al.*, 1985). In the intact male rat, it was concluded that naloxone had no effect on gonadotrophin secretion until the peripubertal period, when testosterone secretion was increasing (Schulz *et al.*, 1982; Bhanot and Wilkinson, 1983; Cicero *et al.*, 1986). It has been suggested that the presence of gonadal steroids may be important for opioidergic control of gonadotrophin secretion (Limonta *et al.*, 1987). In contrast to this, it appears that opioidergic control over LH secretion in ram lambs occurs early when testosterone concentrations are low, is subsequently diminished and then re-established close to puberty (Rawlings *et al.*, 1991). MacDonald *et al.* (1990) studied opioidergic regulation of LH secretion in Holstein bull calves from 3 to 21 weeks of age. Separate groups of animals received a single bolus infusion of the opioid receptor antagonist naloxone at intervals of two to four weeks and its effects on mean circulating LH concentrations were observed over 2 h. They concluded that, except at the onset of the early rise in LH secretion, endogenous opioids inhibited LH secretion up to 21 weeks of age. The aim of our study was to establish the degree of opioidergic control over pulsatile LH and FSH secretion during the early prepubertal period and particularly to document the role of opioids in the regulation of the early transient rise in gonadotrophin secretion in the beef bull calf.

Materials and Methods

Animals

Ten age matched (± 5 days), spring-born, Hereford bull calves were maintained in a beef management system; calves were nursed at pasture until weaning at 21 weeks of age, from which time they were maintained in a paddock and were fed a ration of 21% ground barley, 51% ground hay, 22% ground straw, 0.005% of a 1:1 calcium:phosphorus mineral mix (w/w) *ad libitum*, with brome alfalfa hay and water freely available. Once every two weeks, from 2 to 48 weeks of age, each calf was weighed and a single blood sample collected by jugular venepuncture. Scrotal circumference was also measured once every two weeks from 20 to 48 weeks of age (Hahn *et al.*, 1969).

Naloxone treatments

The ten calves were randomly assigned to either naloxone treatment or saline control groups. At 4, 12, 18, 24 and 32

weeks of age the calves received hourly i.v. injections of naloxone in saline ($n = 5$: 1 mg kg⁻¹ body weight h⁻¹; Research Biomedicals Inc., Natick, MA) or physiological saline solution ($n = 5$) for 12 h. The calves were fitted with jugular catheters (vinyl tubing SV-70; internal and external diameters 1.0 mm and 1.5 mm, respectively; Dural Plastics & Engineering, Dural, NSW, Australia) 18 h before treatment. Throughout the 12 h infusion period, 4 ml blood samples were collected every 12 min. During infusion periods, calves were housed loose in pens and were free to nurse their dams which were tied in stalls. Weaned calves were cannulated in a similar way and housed unrestrained. All blood samples were allowed to clot at room temperature for 12–18 h; the clots were removed and after centrifugation at 1500 g for 20 min, the serum was poured off and stored at -20°C until assayed.

Radioimmunoassays

FSH and LH. Serum concentrations of FSH and LH were determined in double antibody radioimmunoassays (Rawlings *et al.*, 1984). For the FSH assay, the first antibody used was NIDDK-anti-oFSH-I, and FSH concentrations are expressed in terms of USDA-bFSH-II. The range of the standard curve was 0.2–16 ng ml⁻¹. The sensitivity of the assay was 0.2 ng ml⁻¹ (defined as the lowest concentration of unlabelled FSH capable of displacing iodinated FSH from the first antibody; $P < 0.05$, by Students *t* test). Intra- and interassay coefficients of variation (CVs) were 9.1 and 14.0% (mean = 3.10 ng ml⁻¹ serum) and 9.1 and 16.7% (mean = 1.69 ng ml⁻¹ serum), respectively. LH concentrations are expressed in terms of NIDDK-bLH4. The range of the standard curve was 0.1–8.0 ng ml⁻¹. The sensitivity of the assay was 0.1 ng ml⁻¹. Intra- and interassay CVs were 12.3 and 18.7% (mean = 2.90 ng ml⁻¹ serum) and 11.2 and 18.6% (mean = 0.71 ng ml⁻¹ serum), respectively.

Testosterone and oestradiol. Serum testosterone and oestradiol concentrations were measured by radioimmunoassay (Cook *et al.*, 1982; Joseph *et al.*, 1992, respectively). The range of the testosterone standard curve was 0.03–5.00 ng ml⁻¹ and the sensitivity was 0.03 ng ml⁻¹. The intra- and interassay CVs were 9 and 15% (mean = 2.14 ng ml⁻¹ serum) and 9 and 12% (mean = 1.25 ng ml⁻¹ serum), respectively. The range of the oestradiol standard curve was 1–100 pg ml⁻¹ and the assay sensitivity was 1 pg oestradiol ml⁻¹ serum. The intra- and interassay CVs were 14 and 19% (mean = 13.2 pg ml⁻¹ serum) and 14 and 15%, respectively (mean = 6.7 pg ml⁻¹ serum).

Statistical analysis

The episodic patterns of circulating LH and FSH in blood samples collected every 12 min for 12 h were determined using PC-Pulsar (J. Gitzen and V. Ramirez, University of Illinois) and are expressed in terms of mean and basal circulating concentrations, pulse amplitude and pulse frequency. Pulses were identified using standard deviation criteria (Merriam and Wachter, 1982) and basal concentrations were determined by subtraction of the pulses from the 12 h gonadotrophin profile.

For the periods of intensive collection of blood samples, comparison of the gonadotrophin data between control and

naloxone-treated heifers at each age was done by repeated measures ANOVA on the Statistical Analysis System (SAS Version 6, SAS Institute Inc., Cary, NC). Student–Newman–Keuls (SNK) test for least significant difference ($P < 0.05$) was used as the post ANOVA test. The patterns of mean serum concentrations of FSH, LH, testosterone and oestradiol, in blood samples taken every two weeks, were analysed using repeated measures ANOVA (True Epistat®: Epistat Services, Richardson, Texas) and SNK ($P < 0.05$). The linear regression correlation between testosterone and oestradiol was also done using True Epistat®. All the data are presented as means $\pm$ SEM.

Results

The mean ($\pm$ SEM) birth weight of bull calves was 42.0 ± 1.6 kg and the mean age at puberty, as determined by a scrotal circumference of 28 cm (Lunstra *et al.*, 1978), was 37.7 ± 0.7 weeks of age (ranging between 34.6 and 42.6 weeks), at a body weight of 293.1 ± 7.0 kg. Mean growth rate between birth and 40 weeks of age was 7.1 ± 0.2 kg week⁻¹. Naloxone treatments had no effect on the age at puberty ($P > 0.05$).

Repeated measures ANOVA of the annual profiles of the serum concentrations of LH, FSH, testosterone and oestradiol in blood samples taken every two weeks (Fig. 1) all showed a significant effect of age ($P < 0.0002$). The serum LH concentrations at six weeks of age were higher ($P < 0.05$) than 52% of the remaining points and the serum FSH concentrations at 20 weeks were higher ($P < 0.05$) than 83% of the remaining points. Serum concentrations of both testosterone and oestradiol showed a significant increase with age ($P < 0.0001$). Testosterone concentrations from 40 to 48 weeks of age (inclusive) were greater than at all other ages ($P < 0.01$) and similarly for oestradiol, concentrations between 40 and 48 weeks were greater than at 89% of the remaining sampling times ($P < 0.05$). The linear regression coefficient between testosterone and oestradiol was 0.915 ($P < 0.0001$).

In blood samples taken every 12 min for 12 h from the control animals (Fig. 2), mean serum LH concentrations and LH pulse frequency were higher at 12 and 18 weeks than at 4, 24 and 32 weeks of age ($P < 0.05$). LH pulse amplitude decreased from 4 to 18 weeks of age ($P < 0.05$) but basal serum LH concentrations did not change between periods of collection of blood samples ($P > 0.05$). The only effect of age on the pulsatile secretion of FSH in the control animals was that FSH pulse amplitude (Fig. 3) decreased from 4 to 32 weeks of age ($P < 0.05$).

Naloxone treatments increased mean serum concentrations of LH ($P < 0.05$) and LH pulse frequency ($P < 0.01$) and decreased LH pulse amplitude ($P < 0.05$) at four weeks of age (Figs 2 and 4). At 12 weeks of age naloxone increased mean and basal concentrations of LH ($P < 0.01$) and LH pulse amplitude ($P < 0.05$), but had no effect on pulse frequency ($P > 0.05$). At 18 weeks of age naloxone significantly increased only mean serum concentrations of LH ($P < 0.01$); the apparent effect on basal concentrations of LH only approached significance ($P = 0.07$). At 24 weeks, naloxone increased LH pulse frequency ($P < 0.01$), and at 32 weeks naloxone had no significant effect on the pulsatile secretion of LH ($P > 0.05$), but did tend

Fig. 1. Mean ($\pm$ SEM) serum concentrations of (a) LH, (b) FSH, (c) testosterone and scrotal circumference and (d) oestradiol for Hereford bulls from which blood samples were collected every two weeks from 2 to 48 weeks of age ($n = 10$). P = puberty at 37.0 ± 0.7 weeks.

to increase LH pulse frequency ($P = 0.06$). The only significant effect of naloxone on the pulsatile secretion of FSH was to increase FSH pulse frequency at 24 weeks of age ($P < 0.05$; Fig. 3). However, naloxone tended to decrease FSH pulse amplitude at 18 weeks of age ($P = 0.09$) and to increase it at 24 weeks of age ($P = 0.08$).

Discussion

On the basis of samples collected every other week, a transient rise in circulating gonadotrophin concentrations was seen in the early prepubertal period, in agreement with previous observations (Rawlings *et al.*, 1978; Schams *et al.*, 1981). Data from intensive bleedings showed that the increase in mean serum LH concentrations, between 6 and 20 weeks of age, was caused by an increase in LH pulse frequency, despite a decrease in LH pulse amplitude. An increase in LH pulse frequency at this time has been noted previously (McCarthy *et al.*, 1979; Amann and Walker, 1983). A similar trend was seen in FSH data from blood samples collected every two weeks and every 12 min for 12 h at

Fig. 2. (a) Mean, (b) basal serum LH concentrations, (c) LH pulse amplitude and (d) frequency (mean $\pm$ SEM) in bulls treated with naloxone (□; $n = 5$; 1 mg kg⁻¹ body weight h⁻¹) or saline (■; $n = 5$) for 12 h at 4, 12, 18, 24 and 32 weeks of age; blood samples were taken every 12 min for 12 h. Differences between treatment and control groups at an age are shown by * $P < 0.05$ and ** $P < 0.01$. Control groups with different superscripts are significantly different from each other ($P < 0.05$).

4, 12, 18, 24 and 32 weeks of age. It appeared that the mean peak serum concentrations of FSH at 20 weeks of age was too transient to be well reflected in blood samples taken frequently at 18 and 24 weeks of age. The short-lived increase in circulating LH and FSH concentrations between 6 and 20 weeks of age are assumed to be important for the initiation of testicular growth and differentiation (McCarthy *et al.*, 1979; Amann and Walker, 1983). During this early period of development, androstenedione and 5 α -reduced androgens are secreted as well as low levels of testosterone (Rawlings *et al.*, 1972; Abdel Malek Bedair and Thibier, 1979; Rawlings and Cook, 1986). Differentiated Sertoli cells and Leydig cells first appear after 20 weeks of age (Curtis and Amann, 1981) and serum testosterone concentrations start to increase at about 15–20 weeks of age (Rawlings *et al.*, 1978; McCarthy *et al.*, 1979).

The temporal pattern of serum oestradiol concentrations reflected testosterone concentrations in a linear fashion in bull calves during the study reported here. This suggested a similar site and stimulus for production and secretion of both steroids. The relative roles of oestradiol and testosterone as negative feedback agents on gonadotrophin secretion in the bull calf are unclear; both oestradiol and testosterone have potent inhibitory effects on pulsatile LH secretion in bull calves (Schanbacher,

1984), castrated bulls (Schanbacher *et al.*, 1983; Kennedy and Rawlings, 1984) and in castrated rams (D'Occhio *et al.*, 1983). In a previous study, serum concentrations of oestradiol rose in the young bull calf only to fall before the early rise in gonadotrophin secretion (Amann *et al.*, 1986). An increase in serum concentrations of oestradiol from two to six weeks of age was seen in the study reported here but no subsequent decline was observed.

Our data showed that in the four-week-old bull calf there was opioidergic inhibition of LH pulse frequency. Naloxone treatment at this age increased LH pulse frequency and caused a decrease in pulse amplitude. This inverse relationship between frequency and amplitude demonstrated the limited LH content of the pituitary at this time (Amann *et al.*, 1986). The removal of opioidergic inhibition over LH pulse frequency between 4 and 12 weeks of age may have allowed the early rise in LH secretion to occur. During this time, no further increase in LH pulse frequency could be stimulated with naloxone. At between 18 and 24 weeks of age opioidergic inhibition of LH pulse frequency was re-established and mean serum concentrations of LH were decreased. It therefore appeared that the early transient rise in circulating LH concentrations between 6 and 20 weeks of age was at least contributed to by a temporary removal of opioidergic suppression on LH pulse frequency.

Fig. 3. (a) Mean and (b) basal serum FSH concentrations and (c) FSH pulse amplitude and (d) frequency (mean $\pm$ SEM) in bulls treated with naloxone (□; $n = 5$; 1 mg kg⁻¹ body weight h⁻¹) or saline (■; $n = 5$) for 12 h at 4, 12, 18, 24 and 32 weeks of age; blood samples were taken every 12 min for 12 h. Differences between treatment and control groups at an age are shown by * $P < 0.05$. Control groups with different superscripts are significantly different from each other ($P < 0.05$).

MacDonald *et al.* (1990) suggested that opioidergic suppression of mean serum concentrations of LH in Holstein bull calves was absent during the onset of the early prepubertal increase in LH secretion. In their study, treatments consisted of a single bolus injection of naloxone preceded and followed by 2 h of blood sampling. Their study did not extend beyond 21 weeks of age and LH secretion was characterized only in terms of mean serum concentrations. LH pulse frequency at these ages is about one pulse in 2 h or less, and it is known that a challenge with naloxone produces an instant LH pulse in many situations (Currie and Rawlings, 1989). Long periods of naloxone treatment are therefore preferable to determine opioidergic influences on gonadotrophin secretion.

In our study the only parameter of pulsatile LH secretion that appeared to escape opioidergic suppression during the early rise in LH secretion was LH pulse frequency. During this time opioidergic systems suppressed LH pulse amplitude and basal serum concentrations of LH; presumably a fall in opioidergic suppression of LH pulse frequency between 4 and 12 weeks of age caused an increase in pulse frequency which contributed to

the increase in mean serum LH concentrations (McCarthy *et al.*, 1979; Amann and Walker, 1983). Our data also indicated that, despite a lack of opioidergic suppression of mean and basal serum LH concentrations and LH pulse amplitude at 24 and 32 weeks of age, opioidergic suppression of LH pulse frequency could have at least partially explained the drop in mean serum LH concentrations at that time. However, other mechanisms such as enhanced steroid negative feedback must also be important (Schanbacher, 1984).

Prepubertal heifers show a similar early rise in gonadotrophin secretion to bulls (Schams *et al.*, 1981; Evans *et al.*, 1992). In heifers the rise is due to an increase in pulse amplitude. Significant opioidergic inhibition of gonadotrophin secretion occurs only at 4 months of age in heifers (Evans *et al.*, 1992). The argument for a role of endogenous opioids in the early rise in LH secretion in heifers did not appear to be as strong as in the bulls in this study.

Opioidergic control of FSH secretion in the bull calf appeared to be much less than that for LH; the only significant effects of naloxone were seen after the early rise in FSH secretion, in the

Fig. 4. The temporal pattern of circulating concentrations of serum LH in a control calf (●; saline) and a naloxone-treated calf (○; 1 mg kg⁻¹ body weight h⁻¹ on the h) at (a) 4, (b) 12, (c) 18, (d) 24 and (e) 32 weeks of age. Blood samples were collected every 12 min for 12 h.

form of an increase in pulse frequency at 24 weeks of age. Since opioidergic regulation of the pituitary secretion of gonadotrophins occurs via GnRH at the level of the hypothalamus (Blank and Roberts, 1982), the differential response of LH and FSH to naloxone treatments may be due to unequal pituitary stores of the gonadotrophins or differential pituitary secretory patterns, both of which may alter with age. Naloxone has little effect on the pattern of circulating FSH in prepubertal heifers (Evans *et al.*, 1992), mature ewes (Currie and Rawlings, 1989), ewe lambs (Rawlings and Churchill, 1990) or ram lambs (Rawlings *et al.*, 1991). This is in complete contrast to four- to five-week-old male pigs in which i.p. injection of naloxone stimulates FSH but not LH secretion (Trudeau *et al.*, 1990).

In ram lambs opioidergic suppression of LH pulse amplitude occurs only at an early age (5–10 weeks) and is then absent until puberty at 34 weeks of age (Rawlings *et al.*, 1991). In the mature ram it was suggested that pulsatile LH secretion is regulated by endogenous opioids (Schanbacher, 1985) and that

opioids mediate steroid negative feedback suppression of LH secretion. Seasonal changes in opioidergic regulation of LH secretion may occur, indicating that opioids may mediate environmental factors at the level of the hypothalamus (Ebling and Lincoln, 1985). The exact pathways by which opioidergic systems regulate hypothalamic activity are unclear.

Data presented here do not support the idea that opioid pathways convey only steroid negative feedback messages. At 4 and 24 weeks of age, when circulating testosterone concentrations were low, naloxone increased gonadotrophin secretion. At 32 weeks of age when scrotal circumference and testosterone concentrations were increasing, naloxone had no significant effects. This situation may be analogous to the ram lamb in which opioidergic regulation of LH secretion occurred early on when testosterone concentrations were low (steroid independent) and was then absent until puberty, when LH secretion was suppressed by opioids but in the presence of high serum testosterone concentrations (steroid dependent) (Rawlings *et al.*, 1991). Unfortunately, the bull calves in the present study were not challenged with naloxone around the time of puberty. Naloxone has been shown to increase LH secretion in steers (Peck *et al.*, 1988) and to increase LH pulse frequency in castrated and intact rams (Schanbacher, 1985). Hence, opioidergic regulation of LH secretion may have steroid dependent and steroid independent components in prepubertal bulls and rams.

In conclusion we have shown that between 6 and 20 weeks of age there are increased circulating concentrations of LH and FSH in the bull calf and that the removal and subsequent re-establishment of opioidergic inhibition on LH pulse frequency may contribute to the early rise and fall in serum LH concentrations.

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