Non-steroidal factors in bovine follicular fluid inhibit or facilitate the action of pulsatile administration of GnRH on LH release in the female rat

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Abstract

Using steroid-free bovine follicular fluid (bFF), we studied the action of gonadotrophin surge-inhibiting factor/attenuating factor (GnSIF/AF) on GnRH-induced self-priming in phenobarbital-blocked female rats. For the experiments we used intact rats, short-term (4 h) ovariectomized (OVX) rats and long-term (14 days) OVX rats. In the latter case the rats were injected with 17β-oestradiol benzoate (OB, 40 μg) or vehicle only, 2 or 48 h before the experiment. GnRH (10–50 pmol/kg body weight) was injected intra-arterially in 5 or 15 pulses, respectively 60 or 20 min apart, starting 1 or 4 h after injection of bFF (0·5 or 1·0 ml). In response to 25 pmol/kg GnRH pulses (1/h), we observed no effect in the long-term OVX rats, a minor effect in the intact rats and an enhanced self-priming effect in the short-term OVX rats. Administration of bFF attenuated or even completely inhibited the self-priming process. However, in the case of long-term OVX rats LH release was inhibited only after long-term OB priming. Furthermore, 4 h after administration of bFF, LH release in response to 25 pmol/kg GnRH pulses (3/h) was inhibited transiently in intact rats and long-term OVX rats. The results support the hypothesis of a functional antagonistic action between GnRH and GnSIF/AF. However, when injected 1 h before, bFF facilitated the initial release of the surge-like LH pattern in intact rats in response to 3 pulses/h of GnRH. These results are consistent with an important role of GnSIF/AF and other non-steroidal ovarian factors in the control of both low LH concentrations and the generation of the LH surge. Some genomic action of oestradiol might be a prerequisite for the inhibitory effect of GnSIF/AF on GnRH-induced LH release.

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Introduction

Administration of supra-physiological amounts of gonadotrophin-releasing hormone (GnRH) to female rats gives rise to a protein synthesis-dependent increased luteinizing hormone (LH) response of the gonadotrophic cells to GnRH, known as the self-priming process (Edwardson & Gilbert 1975, de Koning *et al.* 1976, Pickering & Fink 1976, Waring & Turgeon 1980, Evans *et al.* 1984, Busbridge *et al.* 1990, de Koning 1995, Fink 1995). The LH surge is the only self-priming effect generated by endogenous GnRH in the rat (Gallo 1981*a*, Padmanabhan *et al.* 1982, Fox & Smith 1985, Allen *et al.* 1988, Kalra 1993). This may be inherent to the physiological role of LH in the optimal maturation of oocytes. For successful fertilization, increases of LH must be prevented until the onset of the LH surge (Mattheij *et al.* 1994, see also de Koning 1995).

The antagonistic interaction between GnRH and the ovarian protein, gonadotrophin surge-inhibiting factor or attenuating factor (GnSIF/AF), may be responsible for this tightly controlled LH release (de Koning *et al.* 1979, 1980, 1987, 1989, Busbridge *et al.* 1990, Knight *et al.* 1990, Koppenaal *et al.* 1993, Fowler *et al.* 1994, de Koning 1995, van Dieten & de Koning 1995, Fowler & Templeton 1996). It was postulated that after each pulse of GnRH, GnSIF/AF neutralizes the potential self-priming action of GnRH before it can become effective, thereby maintaining a state of low LH responsiveness of the gonadotrophs to the action of the next GnRH pulse (de Koning 1995, van Dieten & de Koning 1995). During the LH surge, GnSIF/AF does not sufficiently antagonize the priming effect of the increased GnRH pulse frequency and thus the pituitary LH responsiveness to GnRH will be enhanced.

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Different unique monomeric forms have been demonstrated (Tio *et al.* 1994, Danforth & Cheng 1995). GnSIF/AF may act downstream from cAMP- and diacylglycerol-activated pathways and upstream from mitogen-activated protein kinase activation by GnRH (Helder *et al.* 1997, Tijssen *et al.* 1997). Here, we have studied the acute effects of GnSIF/AF (in steroid-free bovine follicular fluid (bFF)) on the induction of LH release by endogenous-like GnRH pulse patterns.

Materials and Methods

Animals

Adult female Wistar rats were purchased from Broekhuizen (Someren, The Netherlands). They were allowed free access to food and water in an animal room illuminated from 0700 to 1900 h and kept at a constant temperature of 22 °C. Vaginal smears were taken daily and only regular 4-day-cyclic rats were used on the day of pro-oestrus. Animals were subjected to ovariectomy (OVX) either 4 h (short-term OVX) or 14 days (long-term OVX) before the start of the experiment. The weight of the animals at the time of the experiment was 200–240 g. Animal procedures were in compliance with the Guidelines on the Handling and Training of Laboratory Animals (Universities Federation for Animal Welfare), and the local Institutional Animal Care Committee.

Experimental procedures

The rats were provided with a cannula into the carotid artery for injection of GnRH, bFF and control serum, and for blood sampling. In a preliminary experiment 1 ml bFF was injected s.c. or i.p. The long-term OVX rats were injected s.c. with 17β-oestradiol benzoate (OB; 40 μg in 0·1 ml arachidis oil) either 2 or 48 h before the administration of GnRH. Appropriate control rats received arachidis oil only.

On the day of the experiment, phenobarbital sodium (80 mg/kg body weight (BW)) was injected i.p. 1 h before the GnRH administrations and 40 mg/kg BW 2·5 h later. This treatment blocked the preovulatory LH surge and LH concentrations in OVX rats (Schuiling *et al.* 1976). Since phenobarbital also lowers the body temperature, a heating mattress was placed under the cages to maintain normal body temperature of the rats during the experiment (Koppenaal *et al.* 1993).

Rats were injected intra-arterially (i.a.) with 0·5–1·0 ml charcoal-stripped bFF (Slaughterhouse, Bodegraven, The Netherlands) on the morning of the day of pro-oestrus 1–4 h before the pulsatile administration of GnRH (Boehringer, Mannheim, Germany). Control rats were injected with serum from 24 h OVX rats.

GnRH was injected i.a. in doses of 25 or 50 pmol/kg BW on either 5 or 15 occasions 1 h or 20 min apart respectively. The injections started at 1300 h (summer time). Blood samples of approximately 400 μl were collected just before and 20 min after each injection, being the time necessary to reach maximal LH concentrations (Aiyer *et al.* 1974, Pickering & Fink 1976). With three injections per hour, samples were taken at the start of the experiment, 20 min later and then after every hour. After each collection, 400 μl saline were injected into the rat and the cannula was filled with 150 μl heparin (250 IU/ml saline; 150 μl being the volume of the cannula). Blood samples were stored at 4 °C for a few hours and then centrifuged. Plasma was frozen and stored for estimation of LH at a later time point.

Experimental series

Experiment 1 In a pilot experiment the effects of s.c. or i.p. injections of GnSIF/AF bioactivity in 1 ml bFF were tested in phenobarbital-blocked pro-oestrous rats. The effect on LH release was measured by pulsatile infusions of GnRH (50 pmol/kg BW per pulse per hour) 1 h later.

Experiment 2 The antagonistic interaction between GnRH and GnSIF/AF on LH release was studied by measuring the effects of injections of 0·5 or 1·0 ml bFF or control serum on pulsatile infusions of GnRH (25 or 50 pmol/kg BW per pulse per hour) in phenobarbital-blocked pro-oestrous rats. In addition, the effects of elimination of GnSIF/AF after short-term OVX (4 h) were studied on the subsequent LH response to a 25 pmol/kg GnRH pulse regime. One hour before GnRH administration, the rats were injected with 1 ml bFF or control serum.

Experiment 3 The effects of long-term (14 day) OVX on the LH response to a 25 pmol/kg GnRH pulse regime were studied. One hour before GnRH administration, the rats were injected with 1 ml bFF or control serum. Besides these treatments, groups of long-term OVX rats were primed with OB, 2 or 48 h before the injections of GnRH.

Experiment 4 The effects of an increased frequency of GnRH (from 1 to 3 pulses/h) on the LH response were studied. Intact and OB-primed long-term OVX rats were used and they were injected with 1 ml bFF or control serum 1 or 4 h before the start of the GnRH injections.

Hormone analysis

The LH assay was carried out according to de Koning *et al.* (1987). The sensitivity of the LH assay, defined as the amount of standard required to suppress binding of

iodinated LH to 90% of that occurring without unlabelled LH, was estimated as 72 ± 2 (S.D.) pg/tube (n=20). The intra- and interassay coefficients of variation were 8·1 and 11·4% respectively. Specific anti-ovine LH was a generous gift from Dr J Th J Uilenbroek (Erasmus University, Rotterdam, The Netherlands). Rat LH-I-6 and LH-RP-3, kindly provided by the National Institute of Pituitary Hormone Program and National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD, USA), were used for iodination and as standard preparation respectively.

The GnRH (1 pulse/h)-stimulated LH response is expressed as the difference in plasma LH concentration just before and 20 min after the GnRH injection. In cases of 3 pulses/h the absolute plasma LH concentrations are given.

Statistical analysis

Statistical comparisons were made by ANOVA and then by Duncan's multiple comparison test (Steel & Torrie 1960). If the data showed heterogeneity of variance, logarithmic transformation was carried out before the analysis. A difference was considered significant when ANOVA showed significant heterogeneity among all groups and the multiple comparison test gave a value of P<0·05 for the groups concerned.

Results

Effect of the route of administration of bFF

In many studies, FFs have been injected either s.c. or i.p. This pilot study investigated the effectiveness of such injections of bFF to suppress the LH release induced by pulsatile administration of GnRH (50 pmol/kg per hour). Figure 1 shows that s.c. injections of bFF did not affect the GnRH-induced self-priming process, whereas i.p. injections were only partly successful. In the latter case, they had no effect or completely inhibited (four out of ten) the GnRH-induced self-priming process by the last 3 pulses of GnRH. In further experiments, bFF was injected i.a., which gave consistent results.

Effect of bFF on GnRH (1 pulse/h)-induced LH release

In this experimental series the effect of supplementation (by bFF) or elimination (by OVX) of GnSIF/AF on the release of LH induced by GnRH pulses of low amplitude and frequency was studied. These GnRH regimes caused moderately increased, endogenous-like, LH blood concentrations and were most sensitive for changes in GnSIF/AF bioactivity (van Dieten & de Koning 1995). The effects of the 25 and 50 pmol/kg GnRH pulsatile regimes are depicted in Fig. 2a–c. GnRH at 10 pmol/kg did not

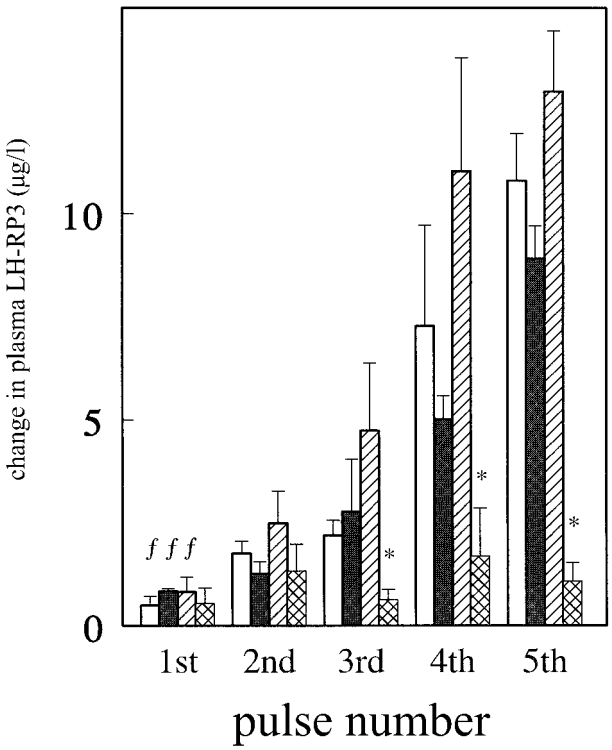

Figure 1 Plasma LH responses (difference between LH concentrations 20 min after each GnRH injection and the LH concentrations immediately before this injection; mean ± S.E.M.) in phenobarbital-blocked intact pro-oestrous rats during five subsequent GnRH injections 1 h apart. GnRH (50 pmol/pulse per kg) was injected i.a. starting 1 h after the bFF injection. One ml injections were given of control blood (open bars; n=14) or of bFF either s.c. (filled bars; n=4) or i.p. (hatched bars). The i.p. group is divided into low (single-hatched bars; n=4) and high (crosshatched bars; n=6) responding subgroups. *f*, the first LH response is significantly lower than that of all subsequent responses of the same treatment group; *, the LH response is significantly decreased by bFF compared with control response within the same pulse-number group. In all cases P<0·05 (Duncan's multiple comparison test).

self-prime the pituitary gland (results not shown). A significant self-priming effect was displayed in response to the 4th and 2nd GnRH pulses of 25 and 50 pmol/kg respectively. Whereas administration of 1 ml bFF completely prevented GnRH-induced self-priming, 0·5 ml bFF delayed the start of the self-priming response by 1 h or attenuated this response to 25 or 50 pmol/kg pulses of GnRH respectively. Short-term OVX rats displayed a faster and more pronounced self-priming effect (Fig. 2c). In addition, 1 ml bFF completely prevented the GnRH self-priming process during the whole experiment.

Role of oestradiol in the action of bFF on GnRH-induced LH release

In the above experiment, GnSIF/AF bioactivity was administered in addition to endogenous GnSIF/AF and

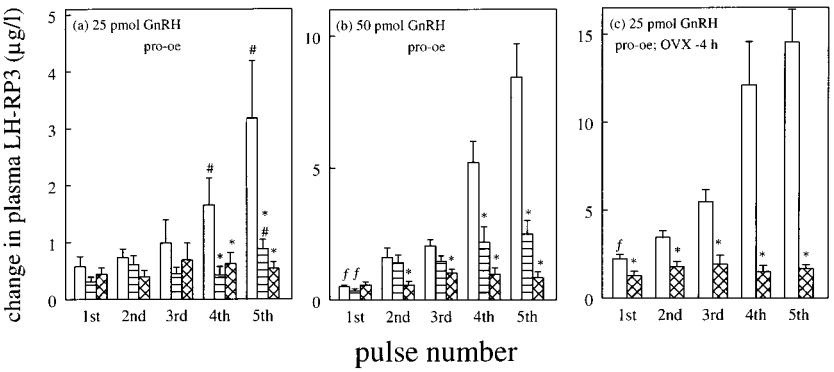

Figure 2 Plasma LH responses (difference between LH concentrations 20 min after each GnRH injection and the LH concentrations immediately before this injection; mean ± S.E.M., n=6) in phenobarbital-blocked (a, b) intact or (c) short-term (− 4 h) OVX pro-oestrous (pro-oe) rats during five subsequent GnRH injections 1 h apart. GnRH was injected i.a. in various concentrations: (a, c) 25 and (b) 50 pmol/pulse per kg. The animals had been pretreated with 0·5 ml (horizontal-hatched bars) or 1 ml (crosshatched bars) injections of bFF or with control blood (open bars) 1 h before the first GnRH pulse. *f*, the first LH response is significantly lower than that of all subsequent responses of the same treatment group; #, the LH response is significantly increased above that of the first response of the same treatment group; *, the LH response is significantly decreased by bFF compared with the control response within the same pulse-number group. In all cases P<0·05 (Duncan's multiple comparison test or Student's *t*-test). Please note different ordinate scales.

other ovarian hormones. To eliminate the ovarian contribution and to study solely the effect of steroid-free bFF, long-term OVX rats were used. Figure 3 shows the results of the effects of OVX on the LH responses to GnRH (25 pmol/kg per hour) and bFF. The self-priming phenomenon was absent in these long-term OVX rats and bFF injections failed to suppress GnRH-induced LH release. In an attempt to restore the action of GnSIF/AF, OB was injected to supplement the oestrogens. When we injected OB 2 h before GnRH administration, LH release was decreased in control and bFF-injected rats, but bFF did not affect GnRH-induced LH release. However, when the rats had been primed with OB 48 h before, bFF now significantly suppressed this release of LH from the

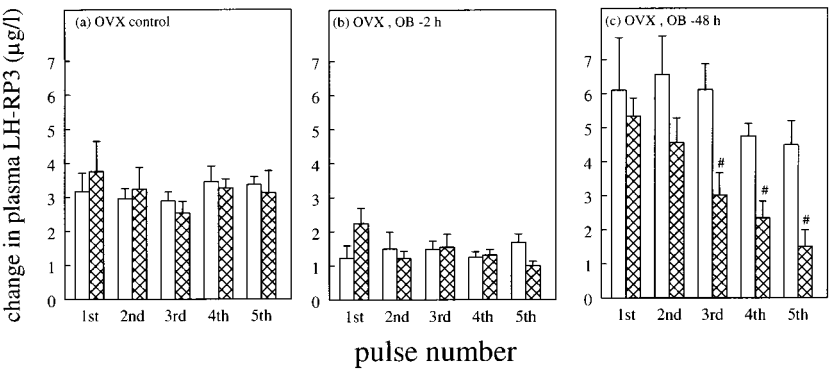

Figure 3 Plasma LH responses (difference between LH concentrations 20 min after each GnRH injection and the LH concentrations immediately before this injection; mean ± S.E.M., n=6) in phenobarbital-blocked rats that had been OVX 14 days before and had received s.c. injections of (a) oil or 40 μg OB in oil either (b) 2 h or (c) 2 days before five subsequent i.a. injections of 25 pmol/pulse per kg GnRH 1 h apart. The animals had been pretreated with 1 ml i.a. injections of bFF (crosshatched bars) or control blood (open bars), 1 h before the first injection of GnRH. #, the LH response is significantly decreased below that of the first response of the same treatment group and that of the control response of the same pulse-number group, P<0·05 (Student's *t*-test).

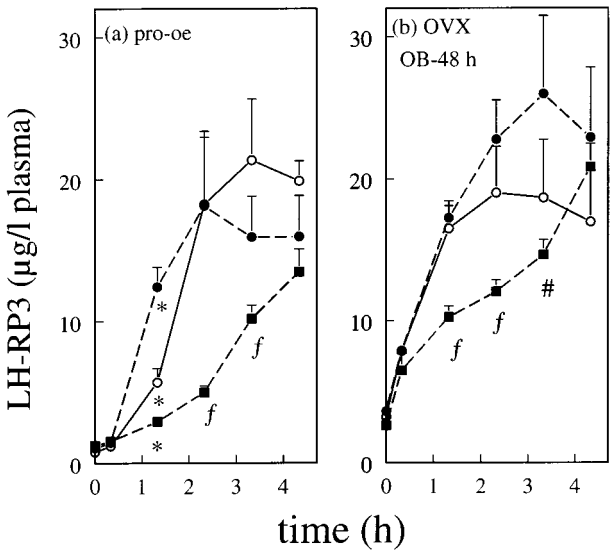

Figure 4 Plasma LH concentrations (mean ± S.E.M., n=4–6) in phenobarbital-blocked (a) pro-oestrous (pro-oe) rats and (b) OVX rats injected s.c. with 40 µg OB 14 days and 2 days before the experiment respectively. The animals received 15 i.a. injections of 25 pmol/pulse per kg GnRH 20 min apart. LH was measured 20 min after the 1st of the 3 pulses of GnRH injected during each hour. The animals had received i.a. 1 ml bFF (dashed lines) 1 h (●) or 4 h (■) before the first pulse of GnRH. Control animals received 1 ml control blood (solid lines, ○). *, at the same time point, the plasma LH concentrations differ significantly; *f*, the plasma LH concentration is significantly lower than that of the other two groups; #, the plasma LH concentration is significantly lower than that of the 1 h bFF group. In all cases P<0·05 (Duncan's multiple comparison test).

3rd stimulation by 25 pmol/kg pulses of GnRH onwards. This decrease was greater than the enhancement of the LH release after OB priming.

Effect of increasing the GnRH pulse frequency on the action of bFF

In the rat, the GnRH pulse frequency increases from about 1 to 3 pulses/h before the onset of the preovulatory LH surge. In view of the antagonistic interaction between GnRH and GnSIF/AF, we studied the effect of bFF on the increased pulsatile administration of GnRH in intact and OB-primed long-term OVX rats. Administration of 1 ml bFF 1 h before the GnRH pulses in intact rats increased the pituitary LH response to GnRH from the 2nd until the 6th pulse of the high-frequency GnRH pulse regime. This initial stimulatory effect of bFF turned into an inhibitory effect when we gave bFF 4 h before the injections of GnRH (Fig. 4a).

In long-term OB-primed OVX rats bFF did not affect the LH release in response to the first 5 pulses of GnRH (Fig. 4b). From the 6th pulse onwards, the bFF-treated group showed a non-significant increase. As in intact rats, when bFF was administered 4 h before the GnRH pulses, they show a decrease in the LH response from about 1–3 h.

Discussion

To study the role of GnSIF/AF, we used bFF to affect the pituitary LH responsiveness to endogenous-like pulses of GnRH. Considering the definition of GnSIF/AF (inhibitors of GnRH-induced LH release), other hormones in bFF, such as inhibin and follistatin, also have intrinsic GnSIF/AF bioactivity (Farnworth *et al.* 1988, Robertson *et al.* 1990, Culler 1992*a*,*b*, Tio *et al.* 1994, Byrne *et al.* 1996). However, distinct pharmacological differences between GnSIF/AF and inhibin bioactivity were observed. First, opposed to inhibin, the liver degrades GnSIF/AF bioactivity (first pass-effect (Uilenbroek *et al.* 1978, de Koning *et al.* 1987)). The present results support this finding by showing the importance of the route of administration of bFF to express the GnSIF/AF bioactivity (Fig. 1). Secondly, it took a 3 day exposure of pituitary cells to purified inhibin and follistatin preparations to cause a more pronounced expression of their intrinsic GnSIF/AF bioactivity (Farnworth *et al.* 1988, Robertson *et al.* 1990). Thirdly, the $t_{1/2}$ of GnSIF/AF and inhibin bioactivity in the blood after i.a. injection of this bFF preparation in OVX rats was about 2 and 1 h respectively (J A M J van Dieten and J de Koning, unpublished results). We chose a design that took advantage of the different pharmacokinetic characteristics between GnSIF/AF and inhibin. The biological effects of GnSIF/AF were studied more explicitly by injecting bFF i.a. shortly before the administration of GnRH.

The present results (Figs 2 and 3) confirm the supposed antagonistic interaction between GnRH and GnSIF/AF; the relative inputs of both components determine the rate of LH release. Increasing the GnRH pulse-frequency (Fig. 4) leads to a preovulatory-like LH surge (Van Dieten & de Koning 1995). Allowing sufficient time (4 h), GnSIF/AF transiently inhibited the LH release in response to the increased pulse frequency of GnRH administration in intact rats as well as in 48 h OB-primed OVX rats. Littman & Hodgen (1984) have demonstrated that circulating GnSIF/AF has a $t_{1/2}$ of 45 min in monkeys. When injected in OVX rats, GnSIF/AF bioactivity in bFF has a $t_{1/2}$ of about 2 h (J A M J van Dieten and J de Koning, unpublished observations). This difference in $t_{1/2}$ may explain the effectiveness of bFF in this study to exert its biological effect even after 4 h.

When bFF was injected 1 h before the increased GnRH pulse regime in intact rats, surprisingly, an increased LH response to GnRH was observed. This effect was not reproduced in the OB-primed OVX rats. Thus, the biological effect of a non-steroidal factor in bFF became unmasked during the lag phase in which the inhibitory

effect of GnSIF/AF was developing. Within 1 h after its administration this factor enhanced the pituitary responsiveness, but only to an increased pulse frequency of GnRH. Thus, not only oestradiol but the specific hormonal environment preceding the LH surge may allow the action of such a factor. Since the GnRH pulse frequency increases during the preovulatory surge in the rat from about 1 to 3 pulses per h (Gallo 1981*a*,*b*, Allen *et al.* 1988, Kalra 1993, van Dieten & de Koning 1995), the results show a possible physiological role for a non-steroidal ovarian factor to contribute to the LH surge (a gonadotrophin surge-facilitating factor).

The present results show that *in vivo* the presence of oestradiol may be a prerequisite for the action of GnSIF/AF. Our results confirm and extend those of Lumpkin *et al.* (1984) and Babu *et al.* (1986), who showed attenuation of GnRH-induced LH release in OB-primed OVX rats by i.p. porcine FF injection(s) administered 6–10 h before. Moreover, the present results prove that the inhibition by bFF exceeds the positive feedback of oestradiol (Fig. 3). Shortly after OVX, bFF still has the potency to suppress GnRH-induced LH release. In long-term OVX rats bFF was not effective when given 2 h after the administration of OB. Thus, it is not the actual presence of oestradiol which is necessary for the effect of GnSIF/AF, but a genomic action that takes some time to develop and to disappear.

Although conflicting data were yielded, the results from *in vitro* investigations also may point to a possible genomic role for oestradiol in the action of GnSIF/AF. In primary pituitary cell cultures, GnSIF/AF bioactivity can be measured without addition of oestrogens into the media. When rat sertoli cell-conditioned medium (rSCM) or bFF inhibited GnRH-induced LH release, there was no additive effect of oestradiol (Massicotte *et al.* 1984, Koppenaal *et al.* 1992). However, rSCM and inhibin reversed the stimulating effect of oestradiol (Massicotte *et al.* 1984, Culler 1992*a*). On the other hand, Byrne *et al.* (1996) showed that oestradiol treatment in the presence of purified GnSIF/AF from human FF enhanced the inhibitory effect of purified human GnSIF/AF. Recently, we showed that the effect of GnSIF/AF bioactivity in bFF was greatest on the days when self-priming was most distinct and oestradiol concentrations were elevated (Tijssen *et al.* 1997). In view of the above, the inconsistent effects of oestradiol may be partly explained by the genomic effects of endogenous oestradiol. If fresh pituitary cells were immediately exposed to GnSIF/AF bioactivity this effect of endogenous oestradiol may have been sufficient to allow the maximal expression of the GnSIF/AF action, whereas if GnSIF/AF was added after 1 or more days, addition of oestradiol may have been needed to restore the (partly) lost genomic effect of endogenous oestradiol.

In conclusion, the present results are the first demonstration of the capacity of GnSIF/AF bioactivity as present in bFF to maintain low LH concentrations in response to an endogenous-like low-dose pulsatile GnRH regime *in vivo*. These results further strengthen a physiological role for endogenous GnSIF/AF in stabilizing low LH concentrations in response to endogenous GnRH. In this process a genomic action of oestradiol may be a prerequisite. Additional ovarian protein factors may be positively involved in the generation of the LH surge, and may respond when the GnRH pulse frequency increases.

Acknowledgements

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