Intracellular antagonistic interaction between GnRH and gonadotrophin surge-inhibiting/attenuating factor bioactivity downstream of second messengers involved in the self-priming process

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The antagonistic control of LH concentrations by GnRH and the putative ovarian factor gonadotrophin surge-inhibiting or -attenuating factor (GnSIF/AF) was studied by perfusion of female rat pituitary glands *in vitro*. LH release and GnRH self-priming were measured in response to five ($0.32\text{--}10\text{ nmol l}^{-1}$) or three (10 nmol l^{-1}) 10 min pulses of GnRH. In the latter case, pulses were preceded by five 10 min pulses of either 1 mmol l^{-1} 8-bromo-cAMP plus 10 mmol l^{-1} theophylline or $1\text{ }\mu\text{mol l}^{-1}$ phorbol 12-myristate 13-acetate. The stimulations were carried out in the presence or absence of steroid-free bovine follicular fluid, which possesses GnSIF/AF bioactivity, to study the effect of GnSIF/AF on the self-priming process during successive stimulations by GnRH. First, the effect of follicular fluid was studied on GnRH-induced LH release from pituitary glands collected during the ovarian cycle. Only when a clear self-priming effect was evident, as on day 2 of dioestrus and the day of pro-oestrus, follicular fluid antagonized the self-priming effect of GnRH. Second, when glands on day 2 of dioestrus were used, self-priming was displayed by various combinations of GnRH and follicular fluid. The pulses with the lowest concentrations of GnRH together with a high concentration of follicular fluid, however, led to stable low amplitude LH pulses. Finally, priming of the pituitary LH response to GnRH with 8-bromo-cAMP plus theophylline or phorbol 12-myristate 13-acetate was inhibited by follicular fluid. These results confirm the control of LH release by GnRH and GnSIF/AF bioactivity. The effect of GnSIF/AF in follicular fluid is most pronounced on the days before the LH surge, showing evidence of its important role in maintaining low LH concentrations during this period. GnSIF/AF neutralizes downstream actions of second messengers involved in GnRH self-priming.

Introduction

Exposure of pituitary glands of female rats to GnRH during the 2 days preceding ovulation induces a very characteristic biphasic LH secretion pattern *in vivo* and *in vitro* (Aiyer *et al.*, 1974; De Koning *et al.*, 1976; Pickering and Fink, 1976; Schuiling *et al.*, 1976). The initial response to GnRH remains low for about 0.5–1.0 h, the time necessary for the synthesis and biological processing of proteins that constitute the rate-limiting step in LH release (De Koning *et al.*, 1976; Pickering and Fink, 1976; Waring and Turgeon, 1980; Evans *et al.*, 1984; Busbridge *et al.*, 1990). After this lag-phase, a GnRH-induced increased secretion of LH is allowed by these newly synthesized pituitary proteins: the GnRH-self-priming effect. It has been hypothesized that during the ovarian cycle

the synthesis of these so-called self-priming-associated proteins (SPAP), or their biological effects, are neutralized by a putative ovarian protein factor. This protein, also named gonadotrophin surge-inhibiting or -attenuating factor (GnSIF/AF) prevents and eliminates endogenous self-priming (De Koning *et al.*, 1979, 1980; Busbridge *et al.*, 1990; Knight *et al.*, 1990; Koppenaal *et al.*, 1991, 1993; De Koning, 1995; Van Dielen and De Koning, 1995; Fowler and Templeton, 1996). In this way, GnSIF/AF and GnRH constitute a functional antagonistic system to stabilize low LH concentrations during the major part of the ovarian cycle (here the effect of GnSIF/AF is relatively stronger), but also to allow the preovulatory LH surge (here the effect of GnRH is relatively stronger; for more details see De Koning, 1995).

Normally, pulsatile administration of GnRH leads to the self-priming effect in phenobarbital-blocked rats. Low physiological concentrations of LH were maintained in such rats by the administration of a low concentration, pulsatile GnRH

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regimen, which demonstrated the effectiveness of endogenous GnSIF/AF for neutralizing the self-priming action of GnRH between these pulses (Van Dieten and De Koning, 1995). In the present study, the antagonistic interaction between GnRH and GnSIF/AF bioactivity (as present in bovine follicular fluid (bFF)) was tested directly at the pituitary by a dynamic *in vitro* design consisting of a perfusion system with hemi-pituitaries. Besides GnRH, agents that mimic the action of the second messengers, cAMP and diacylglycerol (DAG), were also used to induce LH release and the priming effect (De Koning *et al.*, 1981; Turgeon and Waring, 1986; Bourne *et al.*, 1989; Mitchell *et al.*, 1994; Abdilnour and Bourne, 1995).

Materials and Methods

Animals

Adult female rats (200–240 g) from the Wistar-derived colony kept in this laboratory were allowed free access to food and water. The animal room was illuminated from 07.00 to 19.00 h and was kept at a constant temperature of 22°C. Vaginal smears were taken once per day. Only those rats that had at least two regular consecutive 4 day cycles were selected.

Perfusions

Female rats were decapitated between 08.00 and 08.30 h, the pituitary glands were halved and each half was placed in a perfusion chamber. The halves of the same pituitary gland were always divided among different experimental groups. A maximum of eight columns was run at the same time. All perfusion media were supplemented with 0.3% (w/v) bovine serum albumin (FLUKA, Buchs; this prevents adhesions of the reagents and hormones to the inner walls of the perfusion system), 35 mg penicillin ml⁻¹ (Gist-Brocades NV, Delft) and 50 mg gentamicin l⁻¹ (Sigma Chemical Company, St Louis, MO). The pituitary tissues were perfused for 2 h at 37°C with 95% O₂:5% CO₂ saturated TCM 199 (Boehringer, Mannheim). During this time, LH concentrations in the media fell gradually and stabilized at a low baseline concentration (data not shown). The perfusion was then continued with medium to which either bFF (aspirated from follicles of ovaries obtained from the local abattoir and made steroid-free by charcoal treatment; De Koning *et al.*, 1989) or an equivalent amount of charcoal-treated serum from ovariectomized rats was added. If used, bFF and the control serum were present throughout the experiment. If cycloheximide (90 µmol l⁻¹; Boehringer) was used, it was present during and after the GnRH stimulations. Ten minute pulses of GnRH (10 nmol l⁻¹; Boehringer) or of 8-bromo-cAMP (Boehringer) plus theophylline (BrcAMP/theo, 1 mmol l⁻¹ plus 10 mmol l⁻¹; Sigma–Aldrich, Deisenhofen) or phorbol 12-myristate 13-acetate (PMA; 1 µmol l⁻¹; Sigma–Aldrich) were led through the columns. The perfusion chambers were made from 1 ml Primo plastic syringes (ASIK, Rødby) and had a volume of 100 µl. The perfusion velocity was 7.8 ml h⁻¹. Ten minute fractions of media were collected and samples were taken and assayed for LH. Each treatment group consisted of pituitary tissues from at least five rats.

Experimental design

Experiment 1. The effects on LH release from pituitary halves were studied during five successive 10 min pulses of GnRH (0.32, 1.0, 3.2 or 10 nmol l⁻¹) in the absence or presence of 5 ml bFF l⁻¹. Unless otherwise mentioned, in this and the following experiments, pituitary glands were collected on the morning of day 2 of dioestrus. All pulses were separated by 50 min of perfusion with the same medium but without the secretagogue.

Experiment 2. The effects on LH release from pituitary halves were studied during five successive 10 min pulses of 1 nmol GnRH l⁻¹ in the absence or presence of various doses of bFF (1.25, 2.5 and 5.0 ml l⁻¹). In a separate experiment, concentrations of 0.32 and 1.0 nmol l⁻¹ GnRH were used, and the dose of bFF was increased to 10 ml l⁻¹.

Experiment 3. The effects on LH release from pituitary halves were studied during five successive 10 min pulses of 10 nmol GnRH l⁻¹ in the absence or presence of 10 ml bFF l⁻¹. Pituitary glands were collected on the morning of each day of the ovarian cycle.

Experiment 4. The purpose of this experiment was to determine whether the GnSIF/AF bioactivity in bFF could neutralize the effect of second messengers involved in the self-priming action of GnRH. Therefore, pituitary halves were exposed to five successive 10 min pulses of medium only or medium containing BrcAMP/theo (1/10 mmol l⁻¹) or PMA (1 µmol l⁻¹), all in the presence or absence of 10 ml bFF l⁻¹. Their respective effects on priming of the pituitary gland were tested during three successive 10 min pulses of 10 nmol GnRH l⁻¹ with or without the protein synthesis inhibitor cycloheximide. GnRH self-priming is dependent on *de novo* synthesis of proteins which allows the increased rate of LH release. During inhibition of protein synthesis, the initial height of the rate of GnRH-induced LH release should reflect whether or not synthesis of these proteins has already taken place during the 5 h treatment with the various secretagogues. If added, bFF was present throughout the 8 h of perfusion.

Radioimmunoassay of LH

The assay was carried out as described by De Koning *et al.* (1987). The intra- and interassay coefficients of variation were 8.1% and 11.4%, respectively. Specific anti-ovine LH was a generous gift from J. Th. J. Uilenbroek (Erasmus University, Rotterdam). Rat LH-I-6 and LH-RP-3 (both kindly provided by the NHPP, NIDDK, NICHD) were used for iodination and as standard preparation, respectively. The sensitivity of the assays, defined as the amount of the standard required to suppress binding of iodinated LH to 90% of that occurring without unlabelled LH was 72 ± 18 (mean ± S.D.) pg per tube (*n* = 20).

The results are expressed as the total amount of LH–RP3 released h⁻¹ l⁻¹ (area under the curve) in response to each GnRH stimulus, corrected for basal LH release (the area under the curve was calculated from the absolute LH concentration

Fig. 1. The effects on the LH response (mean area under curves; amount of LH-RP3 released $\text{h}^{-1} \text{l}^{-1} \pm \text{SEM}$; $n = 5$) of hemi-pituitaries during five successive 10 min pulses with 0.32, 1.0, 3.2 or 10 nmol GnRH l^{-1} in the absence (□) or presence (■) of 5 ml bovine follicular fluid (bFF) l^{-1} . The pituitary glands were collected on the morning of day 2 of dioestrus. The pulses were separated by 50 min of perfusion with the same medium but without GnRH. †LH response (all pulse numbers) was decreased significantly by bFF; *first LH pulse was significantly lower than all subsequent pulses of the same treatment; ‡pulses were significantly increased compared with the response during the first pulse of the same treatment. In all cases $P < 0.05$ (Duncan's multiple comparison test).

measured during the 10 min before the stimulus and 1 h later). In most cases after GnRH stimulation, LH concentrations returned to basal values within 1 h. However, in response to the two highest concentrations of GnRH, these baseline concentrations remained moderately high.

Statistical analysis

Statistical comparisons were made by analysis of variance and then by Duncan's multiple comparison test (Steel and Torrie, 1960). If the data showed heterogeneity of variance, logarithmic transformation was carried out before statistical analysis. A difference was considered significant when analysis of variance showed heterogeneity for the whole group and the multiple comparison test gave a value of $P < 0.05$ for the two groups concerned.

Results

Experiment 1

The time course effects of five successive 10 min pulses of GnRH (0.32, 1.0, 3.2 or 10 nmol l^{-1}) on LH release from the pituitary gland and the effect of 5 ml bFF l^{-1} are shown (Fig. 1). Each dose regimen of GnRH displayed the self-priming phenomenon. The magnitude of the response was most pronounced with the highest pulse dosages of GnRH. The inclusion of bFF significantly attenuated the first response to GnRH and the self-priming process. Furthermore, it significantly delayed the development of self-priming in response to

the two lowest concentrations of GnRH. At the highest dosages, and only in the absence of bFF, the LH response to GnRH desensitized.

Addition of bFF into the medium did not affect absolute basal concentrations of LH. In both cases these concentrations were $3.5 \pm 0.5 \mu\text{g LH-RP3 l}^{-1}$ (mean $\pm$ SEM; $n = 15$).

Experiment 2

It was investigated whether a concentration of bFF could antagonize completely the self-priming action of a sub-maximally active concentration of GnRH. First, the effect was studied of various amounts of bFF ($1.25\text{--}10.0 \text{ ml l}^{-1}$) on LH release stimulated by five successive pulses of 1 nmol GnRH l^{-1} . The self-priming efficiency of this dose of GnRH decreased when the highest concentrations of bFF were applied (Fig. 2a). GnRH self-priming consistently remained apparent; however, with 5 ml bFF l^{-1} (same data as Expt 1) self-priming was delayed.

In a subsequent experiment, the pituitary glands were exposed to five successive 10 min pulses of 0.32 nmol GnRH l^{-1} in the presence and absence of 10 ml bFF l^{-1} . In this case, GnRH self-priming was completely prevented by bFF and an almost steady low pulsatile release of LH occurred (Fig. 2b). In the control without bFF, GnRH self-priming was still evident.

Experiment 3

The results of the effects of 10 ml bFF l^{-1} and five successive 10 min pulses of 10 nmol GnRH l^{-1} on LH release from pituitary glands collected throughout the ovarian cycle are shown (Fig. 3). They confirm that GnRH self-priming

Fig. 2. The effects on the LH response (mean area under curves; amount of LH-RP3 released $\text{h}^{-1} \text{l}^{-1} \pm \text{SEM}$) of hemi-pituitaries during five successive 10 min pulses with (a) 1 nmol l^{-1} ($n = 5$) or (b) 0.32 ng l^{-1} ($n = 7$) GnRH and 0, 1.25, 2.5, 5 or 10 ml bovine follicular fluid l^{-1} (bFF). The pituitary glands were collected on the morning of day 2 of dioestrus. The pulses were separated by 50 min of perfusion with the same medium but without GnRH. The experiments with the different concentrations of GnRH were carried out separately. †LH responses are significantly decreased by bFF as compared with the time-matched controls; *first LH pulse is significantly lower than all subsequent pulses of the same treatment; ‡pulses are significantly increased compared with the response during the first pulse of the same treatment. In all cases $P < 0.05$ (Duncan's multiple comparison test).

Fig. 3. The effects on the LH response (mean area under curves; amount of LH-RP3 released $\text{h}^{-1} \text{l}^{-1} \pm \text{SEM}$) of hemi-pituitaries during five successive 10 min pulses with 10 nmol GnRH l^{-1} in the absence (□) or presence (■) of 10 ml bovine follicular fluid (bFF) l^{-1} . The pituitary glands were collected on the morning of each day of a 4 day ovarian cycle. The pulses were separated by 50 min of perfusion with the same medium but without GnRH. †On these days the LH responses are decreased significantly by bFF, except the first response on days 1 and 2 of dioestrus; *first LH pulse is significantly lower than all subsequent pulses of the same treatment; ‡pulse is significantly increased compared with the response during the first pulse of the same treatment. In all cases $P < 0.05$ (Duncan's multiple comparison test).

was most pronounced on day 2 of dioestrus and the day of pro-oestrus. On day 1 of dioestrus in both groups, a small but significant self-priming was noticed, whereas at oestrus, no self-priming was displayed. Inclusion of bFF into the media showed significant effects on GnRH-induced LH release only

when GnRH self-priming was most significant, that is on day 2 of dioestrus and the day of pro-oestrus.

The results again demonstrate that, in the absence of bFF, the GnRH pulses desensitize the pituitary to the action of GnRH.

Experiment 4

The effects on the induction of the priming process were studied of five successive 10 min pulses of medium only, or medium with BrcAMP/theo or PMA, all in the presence or absence of bFF (Fig. 4). After the fifth pulse, the LH responses were measured during three additional 10 min exposures to 10 nmol GnRH l^{-1} in the presence or absence of cycloheximide. During the GnRH challenge period, BrcAMP/theo and PMA were not included in the media.

The response to the fifth 10 min exposures of the above agents and the absolute LH concentrations of this response during the last 10 min (Fig. 4a,b) show that neither PMA nor BrcAMP/theo, in the presence or absence of bFF, caused a significant LH pulse. PMA in the presence and absence of bFF significantly increased the absolute LH concentrations, whereas BrcAMP/theo was without effect. This action of PMA was attenuated by the inclusion of bFF into the media.

After 5 h exposure to medium only (Fig. 4c), after the second and third GnRH pulse, a moderate but clear protein synthesis-dependent GnRH self-priming was observed. In agreement with previous experiments (De Koning *et al.*, 1989), the inclusion of bFF into the media decreased the initial phase of low LH release stimulated by GnRH, and thereafter a clear self-priming was displayed. The responses to the third GnRH pulses were no longer significantly different from the control values. In addition, this increase in the rate of LH release was dependent on protein synthesis.

The priming capacity of PMA and of BrcAMP/theo, in the absence of bFF, to the subsequent exposures to GnRH are shown (Fig. 4d,e). PMA was the most effective inducer of priming of the pituitary responsiveness to the first pulse of GnRH. However, the latter effect had disappeared already at the second GnRH pulse. The primed state after BrcAMP/theo treatment remained permanent, but decreased during the concomitant exposure to cycloheximide. This inhibitor of protein synthesis did not significantly affect the action of PMA. The inclusion of bFF into the media blocked the priming effect of BrcAMP/theo on the action of GnRH, but only partly blocked the effect of PMA. However, during the second and third pulse of the GnRH challenge, the self-priming phenomenon was restored. This was most clearly seen after the BrcAMP/theo treatment, when the maximal self-priming effect was reached and, as expected, could be prevented by inclusion of cycloheximide into the media. The GnRH-induced self-priming after the PMA plus bFF treatment was also prevented by inhibition of protein synthesis.

Discussion

Since GnSIF/AF is still not available in sufficient quantities, bFF was used as a substitute for GnSIF/AF bioactivity. Although the results were not always consistent, GnSIF/AF bioactivity may also be an intrinsic feature of two other hormones present in bFF: inhibin and follistatin (Farnworth *et al.*, 1988; Jakubowiak *et al.*, 1989, 1990; Weiss *et al.*, 1993; Tio *et al.*, 1994; see also De Koning, 1995 and Fowler and Templeton, 1996). Two purified preparations of GnSIF/AF were partly characterized, each consisting of a different single polypeptide

chain (Tio *et al.*, 1994; Danforth and Cheng, 1995). Apart from being unique proteins, differences between inhibin and GnSIF/AF bioactivity have been demonstrated. In FSH-treated women, Muttukrishna *et al.* (1994) showed a change in GnSIF/AF bioactivity before a significant change in blood concentrations of immunoreactive inhibin. Animal experiments in which FSH-stimulated GnSIF/AF bioactivity was immunoneutralized by inhibin antibodies exposed the intrinsic GnSIF/AF bioactivity of inhibin (Culler and Negro-Vilar, 1989; Culler, 1992). Studies *in vitro* using purified ovine (Campen and Vale, 1988), bovine (Farnworth *et al.*, 1988), rat (Tio *et al.*, 1994) and recombinant human (Woodruff *et al.*, 1993) inhibin demonstrated that inhibin has some GnSIF/AF-like bioactivity. Thus, GnSIF/AF bioactivity in bFF may stem from different proteins. However, since these proteins are also present in the blood, they may be part of the total circulating GnSIF/AF bioactivity, and may, therefore, contribute to the effect of endogenous GnSIF/AF on the LH responsiveness of the pituitary gland to GnRH.

The small but regular pulsatile LH blood patterns displayed during the major part of the rat ovarian cycle implicate an approximate 1 h frequency of GnRH release (Gallo, 1981a, b; Kalra, 1993). The absence of an endogenous self-priming process in response to the GnRH pulse-generator has been demonstrated (Padmanabhan *et al.*, 1982; De Koning, 1995; Van Dielen and De Koning, 1995). By administration of only small amounts of GnRH once per hour in phenobarbital blocked rats, similar, physiological-like LH pulses were induced. Thus, the action of GnSIF/AF that neutralizes the self-priming effect after each pulse of endogenous GnRH was reproduced. This action of GnSIF/AF keeps the pituitary in an inhibited state to the next pulse of GnRH (Van Dielen and De Koning, 1995).

Use of the present dynamic *in vitro* design shows that GnRH-induced self-priming was antagonized by the inclusion of bFF. Moreover, bFF completely eliminated the self-priming effect of a low concentration pulsatile GnRH regimen, confirming the results *in vivo*. It may be assumed that a similar biological entity in bFF (that is, putative GnSIF/AF) is responsible for the neutralization of the increased LH responsiveness after GnRH exposure *in vivo* and *in vitro* (De Koning, 1995; Van Dielen and De Koning, 1995).

The results show that administration of this factor is effective only on those days when the GnRH self-priming is evident. Stabilization of LH concentrations during the 2 days preceding the LH surge is of the most importance. The inability of bFF to affect GnRH-induced LH release by the pituitary gland early in the cycle may be a consequence of the preceding stimulation by the preovulatory GnRH surge. Similarly, FSH, which may increase the release of GnSIF/AF, is not effective early in the cycle of the rat. A similar insensitivity to the action of GnSIF/AF is found in sheep outside the breeding season (Fowler *et al.*, 1992). It is probable that the oocytes are not susceptible to stimulation by LH during these periods.

In response to the highest concentration of GnRH (10 nmol l^{-1}), and in the absence of bFF, desensitization of LH release occurred. Since the final output of LH release is the result of the various actions of GnRH on self-priming and desensitization, and that of GnSIF/AF, desensitization of the LH response by GnRH may be masked by the ongoing self-priming action of GnRH during incubation with bFF (De Koning *et al.*, 1994). The

Fig. 4. The effects on LH release ($\pm$ SEM; $n = 6$) from hemi-pituitaries taken from rats on day 2 of dioestrus of five successive 10 min pulses with medium only (Med) or medium containing $1 \mu\text{mol}$ phorbol 12-myristate 13-acetate (PMA) l^{-1} or 1 mmol 8-bromo-cAMP l^{-1} plus 10 mmol theophylline (BrCAMP/theo) l^{-1} , all in the absence ($\square$, $\square$) or presence ($\blacksquare$, $\blacksquare$) of 10 ml bovine follicular fluid (bFF) l^{-1} . The pituitaries were challenged by three successive 10 min pulses with medium containing 10 nmol GnRH l^{-1} alone or with $90 \mu\text{mol}$ cycloheximide l^{-1} . In all series the 10 min pulses were separated by 50 min of perfusions with medium of the same content as in the preceding 10 min pulse, but without the secretagogues. If added, bFF was present continuously during the 8 h perfusions. The effects on (a) the LH response during the fifth hour (mean area under curves h^{-1}) or (b) the absolute LH concentrations during the last 10 min of this hour, of hemi-pituitaries treated during five successive 10 min pulses with medium only or medium containing PMA or BrCAMP/theo all without ($\square$) or with ($\blacksquare$) bFF. The effects on the LH response during the challenge of three successive 10 min pulses with medium containing GnRH alone ($\square$, $\blacksquare$) or with cycloheximide ($\square$, $\blacksquare$). The challenges were preceded by five successive 10 min pulses with (c) medium only or (d) medium containing PMA or (e) BrCAMP/theo, all in the absence ($\square$, $\square$) or presence ($\blacksquare$, $\blacksquare$) of bFF. *Significantly different compared with all other treatments; †significantly different compared with the medium control; ‡significantly different compared with the BrCAMP/theo treatment; §first LH pulse is significantly lower than all subsequent pulses of the same treatment; ¶LH pulses after otherwise the same pretreatment are significantly decreased by bFF; †LH pulses after the same pretreatment are significantly decreased compared with cycloheximide. In all cases $P < 0.05$ (Duncan's multiple comparison test).

effect of the latter action is extended, since it takes more time for GnRH to neutralize the inhibitory effect of GnSIF/AF on LH release.

Cyclic AMP and DAG are possible mediators in the protein synthesis-dependent self-priming action of GnRH (De Koning *et al.*, 1981; Turgeon and Waring, 1986; Bourne *et al.*, 1989;

Abdillnour and Bourne, 1995; Mitchell *et al.*, 1994; Fink, 1995). Thus, the present data extend previous results by showing that bFF, and by implication GnSIF/AF bioactivity, not only counteracts the action of GnRH on the self-priming process, but also the priming action of the agents that mimic the action of cAMP and DAG. In the case of DAG, GnRH was able to restore the self-priming action via *de novo* protein synthesis. This points to interactions between GnRH and GnSIF/AF bioactivity, within the GnRH-induced cascade of intracellular processes downstream from cAMP and DAG, on protein synthesis-dependent mediators.

The primed state of the pituitary gland, after treatment with PMA or BrcAMP/theo, decayed sharply within 1 h when cycloheximide was included in the media. Thus, the maintenance of the increased LH response seems to be dependent on the continuous synthesis of protein. This is in contrast to earlier observations when static culture systems were applied (De Koning *et al.*, 1979, 1980, 1981). This difference may point to the disappearance of agents within the perfusion medium and, thus, may indicate autocrine or paracrine interactions of such agents on LH release in the pituitary gland (Weiss and Jameson, 1993).

It should be noted that PMA and BrcAMP/theo have no significant effects on LH release in terms of the LH responses $h^{-1} l^{-1}$. However, PMA, but not BrcAMP/theo and GnRH, causes a sustained increase in the absolute rate of LH release. This increase is blocked by the inclusion of bFF into the media. In agreement with previous observations (De Koning *et al.*, 1981), BrcAMP/theo hardly stimulated the release of LH, but at the same time had a profound priming effect on the pituitary LH responsiveness to subsequent GnRH stimulation. The effect of PMA on the absolute LH concentrations may also explain the initial high LH responsiveness during subsequent stimulation by GnRH 1 h later and its normalization thereafter. However, this decrease may also be caused by the preceding large amounts of LH released. It is clear that bFF, and by implication GnSIF/AF, not only acts by inhibiting the formation of SPAP, but also by blocking the direct releasing action of GnRH downstream of DAG formation.

In conclusion, the present results provide further evidence for a role of the putative GnSIF/AF bioactive entity in controlling low concentrations of LH in the pituitary gland. GnSIF/AF neutralizes the self-priming action of GnRH mediated by the second messengers, cAMP and DAG.

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