Journal of Steroid Biochemistry, Vol. 9, pp. 761–766

0022-4731/78/0801-0761$02.00/0

GONADOLIBERIN-PROMOTED RELEASE OF GONADOTROPINS AND INCREASED SENSITIVITY OF THE PITUITARY BY OESTRADIOL-17β

HENRI KERCRET and JACQUES DUVAL
Laboratoire de Neurobiologie Moléculaire, Equipe de Recherche associée au C.N.R.S. n° 567, Université de Rennes, 35042 Rennes Cedex, France

(*Received* 13 *July* 1977)

SUMMARY

Using perifusion experiments, we were able to distinguish more accurately between the two release processes induced by the gonadoliberin (Gn-RF). Successive short pulses of Gn-RF are followed by similar peaks of LH and FSH release without any potentiation effect. Prolonged infusion of the Gn-RF induces first the acute response and then a sustained high release of both gonadotropins. As a result of this data, a model is proposed in which the amount of gonadotropin, released during the acute response following a Gn-RF pulse, can be taken as a measure of the pituitary sensitivity, whereas that obtained during the permanent infusion can be considered as a measure of the gland capacity to mobilize its gonadotropin pool. The sensitivity to the hypothalamic factor is independent from protein biosynthesis and it is increased by an oestradiol pretreatment. On the contrary, the intense mobilization of the gonadotropin pool is impaired by cycloheximide and unaffected by oestradiol. In our experimental conditions, dihydrotestosterone tends to decrease the sensitivity of the pituitary to the Gn-RF though no significant differences could be registered.

INTRODUCTION

The mechanisms of the action of gonadoliberin (Gn-RF) have not yet been made clear; in particular, it is not clear whether the biosynthesis of any protein is required for gonadotropin release. Although some *in vitro* studies show that the release of LH or FSH is not—or only partly—impaired by inhibitors of protein and RNA biosynthesis [1–4], other results show the contrary [5, 6]. In fact, Gilbert and Edwardson[7] have distinguished between an acute effect of the Gn-RF insensitive to cycloheximide and a self potentiating effect impaired by the antibiotic, results that have been confirmed recently [8, 9]. The search for any *in vitro* modulation by oestrogens or androgens of the LH and FSH cells' responsiveness to the gonadoliberin does not take into consideration these two distinct effects. We thus found it highly desirable to set up dynamic conditions, in which pulses of the Gn-RF could be realized in the presence or absence of steroids and the release of both gonadotropins subsequently registered. First we were led to reinvestigate carefully the bimodal pattern of gonadotropin release

EXPERIMENTAL

Wistar male rats (Centre d'Elevage R. Janvier) were used at 37–40 days old.

Gn-RF promoted gonadotropin release during perifusion

A twin-cell perifusion apparatus built in our laboratory [10] was used. Four hemi-pituitaries were placed in each cell, at 37°C; gassed KRBG (Krebs–Ringer-bicarbonate–glucose solution) circulated from the bottom to the top of the cell at a flow-rate of 12 ml/h, then 4-ml fractions were collected at 0°C into test tubes containing 0.2 ml of the diluting buffer used for radioimmunoassays (including 1% bovine serum albumin). The constant volume within the cells being 0.4 ml, the medium surrounding the glands was entirely renewed in 2 min.

In order to establish exactly the influence of cycloheximide on the 2 steps of gonadotropin release, we determined in 3 preliminary incubations sets the minimum active dose of the antibiotic to be used, the kinetics of protein synthesis inhibition and its effect on the L-fucose attachment to peptide chains (usually the last step in the biosynthesis of glycoproteins and hence of LH and FSH). Using [^{3}H]-valine and [^{3}H]-fucose as precursors, we observed that 10 μg of cycloheximide/ml medium is enough to block the protein biosynthesis in 5 min at the ribosomal level up to 95% whereas the attachment of the sugar moiety to the previously synthetized chain is impaired by less than 5%. We thus used this concentration of antibiotic in the subsequent experiments.

First experiment. At 80 min intervals timed from the start of the perifusion, the Gn-RF was injected into both cells at a concentration of 100 ng/ml over a period of 10 min. Immediately before the 2nd injection, one of the cells was subjected to a 5 min pulse of cycloheximide. The Gn-RF infusion was stopped 2 h 40 min later and only the injection of KRBG was prolonged for a further 80 min.

Steroid effect on the Gn-RF promoted acute gonadotropin release

Oestradiol-17β or 5α-dihydrotestosterone, previously dissolved in the minimum amount of absolute ethanol before dilution with KRBG, was continuously injected into the sample cell while KRBG plus the same amount of ethanol was injected into the control cell. 2 h 20 min after the start and then at 80 min intervals, both cells were submitted to 10 min pulses of Gn-RF at a concentration of 100 ng/ml.

Estimation of LH and FSH

The collected fractions were spun at 40 000 *g* to eliminate all particulate material before gonadotropin determination. The radioimmunoassays were performed using the kits for rat LH and rat FSH supplied by the National Institute for Arthritis and Metabolic Diseases. The iodination of the hormone and the 1st precipitation were carried out according to NIAMD procedure; the 2nd precipitation was done using polyethyleneglycol 6000 (PEG), a fast and inexpensive method which we have recently described [10].

Statistical evaluation

An analysis of variance was used for the data concerning the effect of successive Gn-RF pulses on control glands; a *t*-test was applied to the differences between pairs of steroid-treated and control hemipituitaries.

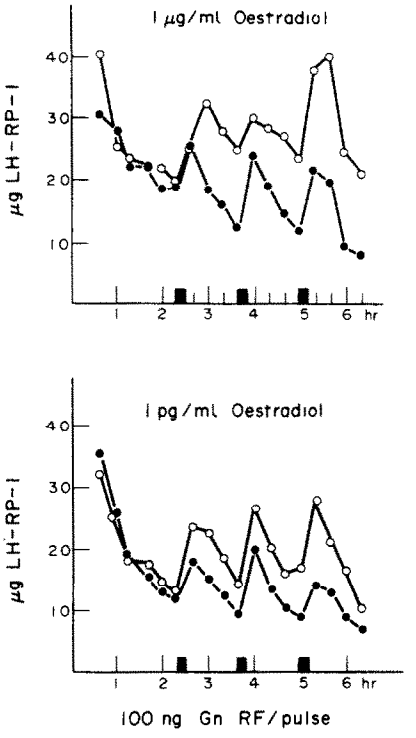

Fig. 1. Influence of oestradiol on the LH release promoted by successive pulses of Gn-RF. 3 × 10 min pulses of 100 ng Gn-RF/ml medium (black rectangles) have been performed; 4 hemi-pituitaries per cell; flow-rate: 12 ml/h. Solid circles—control cell, superfused with KRBG + ethanol. Open circles—sample cell, superfused from time 0 with KRBG + ethanol + oestradiol at the dose indicated.

RESULTS

Cycloheximide action on gonadotropin release

Figure 1 shows the results of the LH release arising from successive 10 min pulses of 100 ng Gn-RF/ml medium (control cells). At first, a very high non specific release was observed during the first 2 h; Thereafter, the base line was usually very good. As expected, the pituitaries gave an immediate discharge following the Gn-RF infusion (the same holds for FSH). The amount of LH and FSH released were estimated for each pulse after subtraction of the non specific output of hormone.

An analysis of variance of the data from the 8 independent experiments reported in Tables 1 and 2 give the following: LH between pulses $F(2.21) = 0.44$. FSH between pulses $F(2.21) = 0.31$.

It follows that the successive pulses of Gn-RF elicit independent secretory responses of the pituitaries; hence, no self-potentiating effect of the hypothalamic factor can be registered.

Figure 2A represents the releases of LH and FSH resulting from a continuous infusion of 10 ng Gn-RF/ml. The response is biphasic: during the 1st hour, LH and FSH surges occurred in the same way as in the pulse experiments; during the 2nd hour, the gonadotropin secretion increased, reached a plateau and then dropped to the base-line as soon as the Gn-RF was withdrawn.

We analyzed the effect of a 5 min pulse of cycloheximide on the successive steps mentioned above. The antibiotic was shown not to alter the LH and FSH releases resulting from a subsequent pulse of Gn-RF (results not given in detail) whereas it immediately impeded the high release usually observed during the continuous infusion of the hypothalamic factor (Fig. 2B). Though the antibiotic was quickly eliminated from the surrounding medium, the releases were only partly reinitiated.

Steroid effects or gonadotropin release

Oestradiol or dihydrotestosterone was continuously infused during the experiment at the various concentrations reported in the Tables 1 and 2. Three successive Gn-RF pulses were given; the amounts of gonadotropins released were estimated for each pulse after subtraction of the respective base line values. It can be seen (Fig. 1) that oestradiol slightly increases the basal output whatever the dose (only 2 doses represented); the androgen has no effect. The analysis of data from Table 1 shows that an oestradiol pretreatment increases the acute LH and FSH secretory responses of the pituitary to the Gn-RF, whatever the pulse numbers or the the dose of steroid. When the glands are superfused with dihydrotestosterone (data from Table 2), a decrease is frequently observed, but the results are not statistically significant.

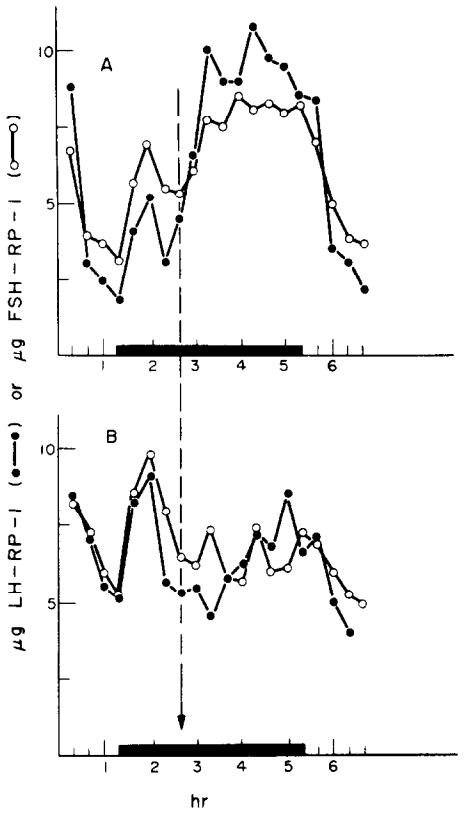

Fig. 2. Influence of cycloheximide during the continuous infusion of Gn-RF. A—Control cell. Gn-RF (10 ng/ml) was infused between 1 h 20 min and 5 h 20 min (black bar). B—Sample cell. Gn-RF (10 ng/ml) was injected as in control cell; a 5 min pulse of cycloheximide was realized at the time indicated by an arrow.

When the Gn-RF is continuously infused in the presence of oestradiol, the release pattern is the same as in Fig. 2, and the total output of LH in the medium during that period does not differ from the amount released in the absence of the steroid (results for 2 experiments: control 45.2 and 55.5 μg LH-RP-1/4 h; oestradiol-treated 45.5 and 66.7 μg LH-RP-1/4 h).

DISCUSSION

The perifusion system clearly enables a dynamic study of gonadotropin release to be performed. Two hours after the start of KRGB infusion, the high non-specific release of LH and FSH ends and successive 10 min pulses of Gn-RF promote LH and FSH surges lasting about 60 min; this corroborates in this respect, previous results [11, 12], but the so-called self-potentiating effect described in female rat pituitaries was not observed [13]. It was further shown that when the glands are submitted to the continuous action of the releasing factor, a high sustained release follows the acute step. In order to explain these observations, we now propose a simplified model (Fig. 3) that precisely shows what is usually called "self-potentiation" by several groups [8, 9, 13].

The hormone released during the acute response is contained in granules linked—or in close vicinity—to the plasma membrane (Fig. 3b); due to the rapid dissociation of the Gn-RF from its membranous receptors [14], successive pulses are independent and do not modify the membrane response. When Gn-RF is permanently renewed, a second process occurs which we interpret as an intense mobilization of the cellular LH and FSH pools (Fig. 3c); whether this mobilization is only a consequence of the sustained output of the hormones, or results from an intracellular action of the Gn-RF remains to be shown.

When the time intervals between pulses were shortened such as in [13], the secretory response was close to what is observed during a continuous infusion, the mobilization of the gonadotropin pools being progressively increased. When glands are incubated in the presence of the hypothalamic factor [8], the results of both steps are sequentially registered

Table 1. Influence of oestradiol-17β on the releases of LH and FSH promoted by successive pulses of Gn-RF

Oestradiol ng/ml		LH (μg/80 min) Pulse no. 1	LH (μg/80 min) Pulse no. 2	LH (μg/80 min) Pulse no. 3	FSH (μg/80 min) Pulse no. 1	FSH (μg/80 min) Pulse no. 2	FSH (μg/80 min) Pulse no. 3
10^{-3}	T	1.40	1.95	1.45	3.15	4.70	2.75
	E_2	3.30	3.65	3.40	3.25	3.50	3.90
10^{-1}	T	3.15	1.75	2.90	2.70	4.35	3.55
	E_2	3.55	3.40	3.90	4.10	5.10	4.30
10	T	—	2.50	3.10	—	3.25	4.40
	E_2	3.90	3.10	3.40	2.05	5.55	5.50
10_3	T	1.50	2.50	2.45	3.15	2.15	3.00
	E_2	3.10	2.85	4.15	3.90	3.40	3.65
$m \pm \sigma$	T	2.02 ± 0.98	2.18 ± 0.38	2.48 ± 0.74	3.00 ± 0.26	3.61 ± 1.15	3.43 ± 0.73
	E_2	3.46 ± 0.34	3.25 ± 0.35	3.71 ± 0.38	3.33 ± 0.92	4.39 ± 1.10	4.34 ± 0.82
t-test		$t(3) = 5.89$ $P < 0.01$			$t(3) = 3.79$ $P < 0.05$		

4 hemi-pituitaries per cell were submitted to successive 10 min pulses of Gn-RF at a concentration of 100 ng/ml. The first pulse occured 2 h 20 min after the start of KRBG (T) or KRBG + oestradiol (E_2) perifusion; interval between pulses: 80 min LH-RP-1 and FSH-RP-1 used as standards.

Table 2. Influence of DHT on the release of LH and FSH promoted by successive pulses of Gn-RF

DHT ng/ml		LH (μg/80 min) Pulse no. 1	2	3	FSH (μg/80 min) Pulse no. 1	2	3
10^{-3}	T	4.80	3.50	5.20	3.40	2.55	3.23
	DHT	4.00	2.75	4.55	2.83	1.70	2.40
10^{-1}	T	3.18	5.15	3.53	1.95	3.70	2.00
	DHT	2.33	4.18	2.43	1.95	2.50	1.68
10^{3+}	T	—	5.65	5.75	—	2.98	2.88
	DHT	4.90	3.70	5.75	2.58	2.18	3.98
10^{3}	T	2.45	3.80	2.80	3.24	2.35	2.88
	DHT	2.45	4.90	2.60	2.60	3.63	2.78
$m \pm \sigma$	T	3.48 ± 1.20	4.53 ± 1.04	4.32 ± 1.38	2.86 ± 0.80	2.90 ± 0.60	2.75 ± 0.52
	DHT	3.42 ± 1.25	3.88 ± 0.90	3.83 ± 1.60	2.49 ± 0.38	2.50 ± 0.82	2.71 ± 0.96
t-test		$t(3) = 2.38$ $P < 0.1$			$t(3) = 1.07$ $P < 0.5$		

Same experiment as described in Table 1, oestradiol being replaced by DHT. ($+10^{3}$ ng/ml instead of 10 ng/ml).

leading to an apparent self-potentiation. This model holds good at least for the male rat since the female pituitary may respond in another fashion due to its different endocrine status. An alternative explanation would be that the Gn-RF progressively increases the number of its own receptors, but it is difficult to understand why the 100 ng/ml pulses of Gn-RF do not have any effect; furthermore, the 5 min cycloheximide pulse (see Fig. 2) would probably be as inefficient on this new set of receptors as in the 1st step of release.

In our opinion, the acute release following a short pulse could be considered as a measure of the sensitivity of the gland whereas the sustained release would represent an estimation of the overall capacity of the pituitary. Indeed, in agreement with others [8, 9, 13], we observed that the acute response is unaltered by cycloheximide whereas the 2nd step is severely impaired. Therefore, our interpretation differs slightly from that of de Koning *et al.*[8]; they claim that during the 1st phase, the Gn-RF promotes the biosynthesis of a protein which has the low turnover required for the 2nd step, enough molecules being already present in the glands at the beginning of the experiment. If this is the case, a 5 min pulse 80 min after the start of Gn-RF infusion (Fig. 2) would not exert any immediate effect; on the contrary, it is extremely efficient. We conclude that either a specific protein with a very high turnover rate is required only for this 2nd step, which appears unlikely, or that by a still unknown process the antibiotic blocks the mobilization of the hormone pool.

Concerning the action of oestradiol on the Gn-RF-induced gonadotropin release, numerous *in vivo* experiments have revealed a negative or positive feedback effect according to the conditions, but the results of *in vitro* experiments are still scarce and sometimes conflicting; Spona[15] describes an increased LH release in a 4 h incubation system in the presence of oestradiol while FSH remains unaltered; the same steroid is shown to slightly stimulate the basal release of both gonadotropins and to diminish the Gn-RF–stimulated release in pituitary cell culture [16, 17]. Two recent reports appear to be contradictory: on the basis of 8 h incubation experiments, de Koning *et al.*[18] claim that oestradiol first inhibits and then increases the pituitary response to Gn-RF; in adenohypophyseal cultured cells, Drouin *et al.*[19] show only an increased sensitivity of the LH response to the Gn-RF without any modification of the maximal

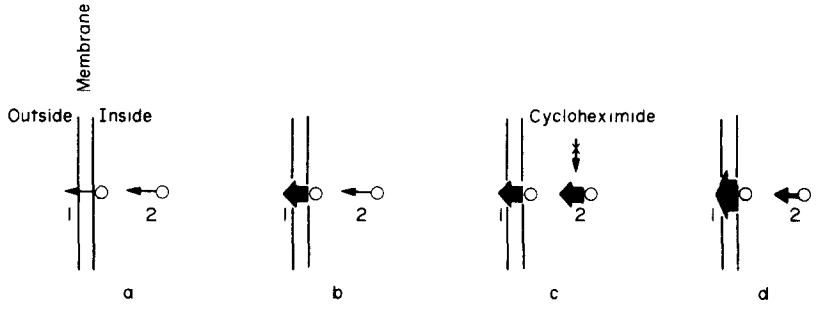

Fig. 3. Model for the acute release [1] and mobilization of the gonadotropin pool [2] through various Gn-RF treatments. a—basal rate of secretion: no Gn-RF. b—rate of secretion after a short pulse of Gn-RF. c—rate of secretion after a continuous infusion of Gn-RF. d—rate of secretion after a pre-treatment with oestradiol followed by a short Gn-RF pulse.

response; however, this effect can only be measured after 10 h of incubation in the presence of the steroid. We also observed a slight increase of the basal gonadotropin secretion (Fig. 1); it can be further observed that oestradiol increases the sensitivity of the pituitary to pulses of the hypothalamic factor, whereas the overall capacity does not appear to be modified. It is certainly possible that we missed the early inhibitory step since our 1st Gn-RF pulse arises 2 h after the beginning of steroid infusion. Despite this fact, our results—apart from the time required for the oestrogen to take effect—concur quite well with those concerning only LH reported by Drouin *et al.*[19]. The steroid either modifies the Gn-RF interaction with the plasma membrane as suggested by Spona [20, 21] or slightly increases the number of granules in the vicinity of the membrane, which would also explain the slight increase of the basal release (see Fig. 3d).

Concerning the androgens, we found no significant effect though a slight decrease was usually observed. Kao *et al.*[22] reported a preliminary stimulation of LH and FSH release followed by a slight inhibition; Drouin and Labrie[23] have also shown a decrease in the sensitivity of the release mechanisms in the LH-secreting cells due to androgens, but in their cell cultures, the steroid has to be present for 40 h before the stimulation in order to get the maximal inhibition; obviously, in our perifusion system, the androgen effect is smaller due to the shorter duration of its action.

Acknowledgements—The skilful technical assistance of Mrs J. Sorais and Mr J. Commandoux has been greatly appreciated. The authors also wish to thank Dr Parlow and the NIAMD, Dr Blanc and the INRA, Dr Enzmann and Farbwerke Hoechst respectively for their gifts of hormones, immun-serum and Gn-RF.

Note added in proof—while this manuscript was being submitted, a report by Hopkins (Hopkins C. R. *J. biophys. Biochem. cytol.* **73**, (1977) 685–695) appeared which describes an analogous biphasic release pattern of LH under the influence of a continuous infusion of Gn-RF through a column filled with immobilized pituitary cells.

REFERENCES

1. Samli M. H. and Geschwind I. I.: Some effects of the hypothalamic luteinizing hormone releasing factor on the biosynthesis and release of luteinizing hormone. *Endocrinology* **81** (1967) 835–848.
2. Jutisz M. and de la Llosa M. P.: Studies on the release of follicle-stimulating hormone *in vitro* from rat pituitary glands stimulated by hypothalamic follicle-stimulating hormone-releasing factor. *Endocrinology* **81** (1967) 1193–1202.
3. Jutisz M. and de la Llosa M. P.: Recherches sur le contrôle de la sécretion de l'hormone folliculo stimulante hypophysaire. *Bull. Soc. Chim. biol.* **50** (1968) 2521–2532.
4. Crighton D. B., Watanabe S., Dharival A. P. S. and McCann S. M.: Failure of inhibitors of protein synthesis to affect the LH-releasing action of hypothalamic extracts *in vitro*. *Proc. Soc. exp. Biol. Med.* **128** (1968) 537–540.
5. Watanabe S., Dharival A. P. S. and McCann S. M.: Effect of inhibitors of protein synthesis on the FSH-releasing action of hypothalamic extracts *in vitro*. *Endocrinology* **82** (1968) 674–684.
6. Ishikawa H., Nagayama T. and Niizuma K.: The mechanism of the stimulation of LH production by synthetic LH-releasing hormone (LH-RH) in tissue culture. *Biochem. biophys. Res. Commun.* **55** (1973) 492–498.
7. Gilbert D. and Edwardson J. A.: The self-potentiating effect of luteinizing hormone-releasing hormone (LH-RH): dose response studies with LH-RH and cycloheximide. *Acta Endocr., Copenh.* Suppl. **199**, (1975) p. 260.
8. De Koning J., Van Dieten J. A. M. J. and Van Rees G. P.: LH dependent synthesis of protein necessary for LH release from rat pituitary glands *in vitro*. *Molec. cell. Endocr.* **5**, (1976) 151–160.
9. Pickering A. J. M. C. and Fink G.: Priming effect of luteinizing hormone releasing factor: *in vitro* and *in vivo* evidence consistent with its dependence upon protein and RNA synthesis. *J. Endocr.* **69** (1976) 373–379.
10. Kercret H. and Duval J.: Utilisation du polyéthylène glycol pour la précipitation du complexe hormone-anticorps dans le dosage radioimmunologique des gonadotrophines de rat. *Biochimie* **58** (1976) 1417–1418.
11. Osland R. B., Gallo R. V. and Williams J. A.: *In vitro* release of luteinizing hormone from anterior pituitary fragments superfused with constant or pulsatile amounts of luteinizing hormone releasing factor. *Endocrinology* **96** (1975) 1210–1216.
12. Kilpatrick M. J., Collins W. P. and Newton J. R.: Studies on the release of gonadotrophins during the superfusion of isolated rat pituitaries in a continuous flow system. *J. Reprod. Fert* **46** (1976) 25–30.
13. Edwardson J. A. and Gilbert D.: Application of an *in vitro* perifusion technique to studies of luteinizing hormone release by rat anterior hemi-pituitaries: self-potentiation by luteinizing hormone releasing hormone. *J. Endocr.* **68** (1976) 197–207.
14. Sternberger L. A. and Petrali J. P.: Quantitative immunocytochemistry of pituitary receptors for luteinizing hormone-releasing hormone. *Cell Tiss. Res.* **162** (1975) 141–176.
15. Spona J.: LH-RH actions modulated by sex steroids. *Endocr. Exp.* **8** (1974) 19–29.
16. Steinberger A. and Chowdhury M.: Effect of testosterone and estradiol on the basal and LRF-stimulated secretion of gonadotropins in pituitary cell culture. *Endocr. Res. Commun.* **1** (1974) 389–401.
17. Tang L. K. L. and Spies H. G.: Effects of gonadal steroids on the basal and LRF-induced gonadotropin secretion by cultures of rat pituitary. *Endocrinology* **96** (1975) 349–356.
18. De Koning J., Van Dieten J. A. M. J. and Van Rees G. P.: Inhibitory and augmentative effects of oestradiol on LH-RH induced release of LH by anterior pituitary glands from intact female rats *in vitro*. *Molec. cell. Endocr.* **5** (1976) 321–330.
19. Drouin J., Lagacé L. and Labrie F.: Estradiol-induced increase of the LH responsiveness to LH releasing hormone (LH-RH) in rat anterior pituitary cells in culture. *Endocrinology* **99** (1976) 1477–1481.
20. Spona J.: LH-RH interaction with the pituitary plasma membrane is affected by sex steroids. *FEBS Lett.* **39** (1974) 221–224.
21. Spona J.: Sex steroids influence LH-RH-receptor interaction. *Endocr. Exp.* **9** (1975) 167–176.
22. Kao L. W. L. and Weisz J.: Direct effect of testosterone and its 5α-reduced metabolites on pituitary LH and

FSH release *in vitro*: change in pituitary responsiveness to hypothalamic extracts. *Endocrinology* **96** (1975) 253–260.

23. Drouin J. and Labrie F.: Selective effect of androgens on LH and FSH release in anterior pituitary cells in culture. *Endocrinology* **98** (1976) 1528–1534.