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GnRH Receptors and GnRH Endocrine Effects on Luteoma Cells

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An ovary implanted into the spleen of an ovariectomized rat develops into a luteinized tumor, growing in response to gonadotrophins. Previously, it was shown that in vivo Buserelin, a gonadotrophin-releasing hormone (GnRH) analog, inhibited tumor growth. To determine if GnRH had a direct effect on tumor cells, the presence of GnRH receptors as well as the endocrine effects of buserelin were studied on tumoral tissue. GnRH receptors were present in luteoma in similar concentrations and dissociation constant (Kd) to control estrous ovaries. In vivo treatment with buserelin did not modify luteoma GnRH receptors. In organ incubations, luteoma secreted significantly higher estradiol and lower progesterone than estrous ovaries; addition of buserelin did not modify steroid secretion. The same difference in basal steroid secretion between luteoma cells and luteal cells superovulated prepubertal ovaries was observed in cell cultures. Although luteinizing-hormone (LH)-stimulated progesterone in both kinds of cells, buserelin significantly inhibited LH-stimulated progesterone only in luteoma cells. These results describe clear differences in basal steroid secretion between tumoral and normal tissue. Furthermore, they show that luteoma possess GnRH receptors similar to those in normal ovarian tissue, and that GnRH analogs have endocrine effects on these cells. Therefore, a direct effect of buserelin on luteoma cells can be postulated.

Key Words: Luteoma; GnRH receptors; buserelin effects; progesterone; estradiol.

Introduction

Previous works have shown that an ovarian tumor develops when an ovary is autotransplanted into the spleen of a bilaterally ovariectomized rat *(1–4)*. This grafted ovary grows in response of gonadotrophin hyperstimulation, and it has been histologically defined as a luteoma as it is formed mostly by luteinized tissue. The authors' data (not shown) and those of others *(5)* demonstrate that it does not appear to malignize at least after 1 yr of growth. Results from the authors' laboratory showed that chronic treatment of tumor bearing rats with buserelin, a gonadotrophin-releasing hormone (GnRH) analog, is effective in inhibiting tumor growth and in suppressing hormone secretion *(6)*. GnRH and its analogs, administered chronically, act at the hypophyseal level downregulating gonadotrophin-releasing hormone receptors and thus inhibiting luteinizing-hormone (LH) and follicle-stimulating hormone (FSH) release. Although this blockage of gonadotrophin secretion is a major factor inducing tumor regression, as tumor growth was shown to depend on LH and FSH stimulation *(4,7,8)*, a direct effect of buserelin on tumoral tissue cannot be disregarded. Extrapituitary actions of GnRH have been extensively reviewed and show an important role of this peptide in the regulation of the rat ovary *(9,10)*. GnRH receptors and a GnRH-like peptide and their respective RNAs have been described in the normal ovary *(11–18)*. Furthermore, ovarian GnRH has been postulated to modulate various local functions such as oocyte maturation and ovulation *(19)*, ovarian steroid hormone synthesis and secretion *(20–23)*, inhibin synthesis *(24,25)*, prostaglandins production *(26)*, and gonadotrophin and PRL receptor levels *(9,27,28)*.

The present set of experiments was designed to evaluate if this experimental ovarian tumor contained GnRH receptors and if GnRH acting directly on this tissue modified hormone secretion. A comparative study of GnRH receptor levels and GnRH endocrine effects in tumoral tissue and in two kinds of control luteal tissue was undertaken.

Results

GnRH Receptors in Ovarian Tissues and Pituitaries from Luteoma Bearing Rats and Control Estrous Rats

Figure 1 shows histology of ovaries. According to Scatchard analysis, ovarian tissues showed a single class of high affinity binding sites (Fig. 2). Kd values were similar among the groups (estrous ovaries: $2.3 \times 10^{-11} M$, tumor: $2.4 \times 10^{-11} M$, tumor-bus: $3.7 \times 10^{-11} M$) and were very similar to Kd values at the pituitary, as has been previously described *(11,29)*. No significant differences in GnRH receptor numbers, determined by saturation analysis, were observed between tumoral and control ovarian tissues. Buserelin treatment for one month in tumor bear-

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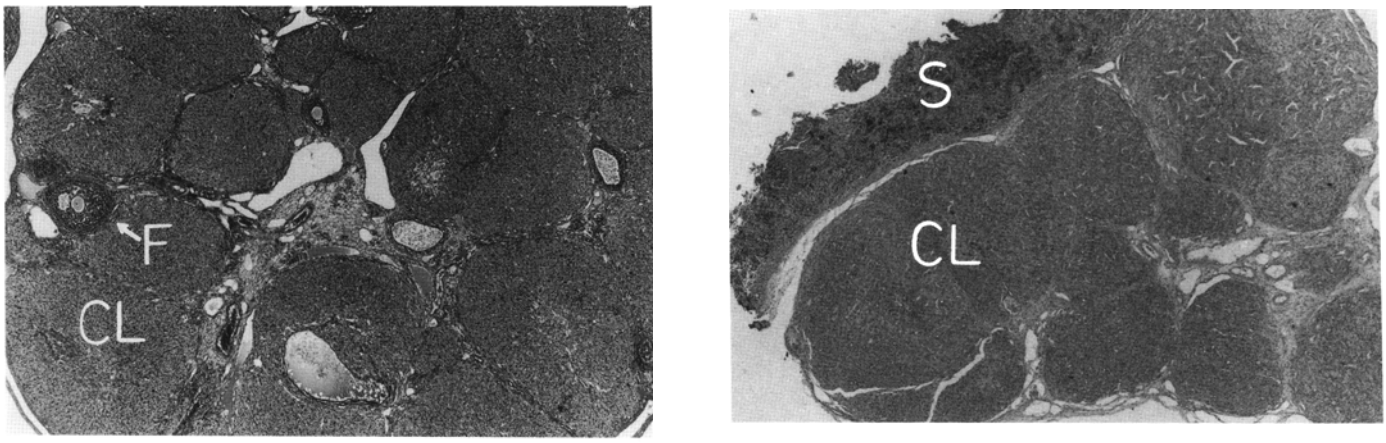

Fig. 1. Histology of prepubertal superovulated ovaries (left) and 1–2 mo-old luteoma (right). CL: corpora lutea, F: follicles, S: spleen; (H&E staining, final magnification: ×40).

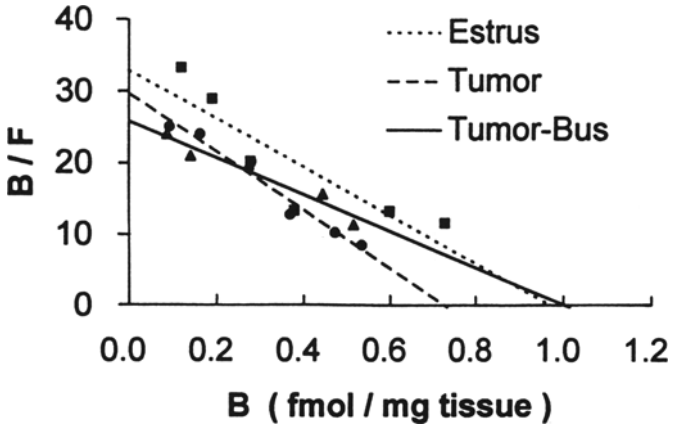

Fig. 2. Scatchard analysis of GnRH binding to ovarian tissues (one experiment representative of three). For this and the following figures and tables: Estrus: ovaries from estrous rats (■.....■); Tumor: luteoma from tumor bearing rats (●.....●); Tumor-bus: luteoma from tumor bearing rats treated with GnRH analog (buserelin) (▲—▲).

Table 1
Ovary and Pituitary GnRH Receptors: B_{max} (fmol/mg tissue)

	Estrus	Tumor	Tumor-bus
Ovarian tissues	0.599 ± 0.119 (9)	0.347 ± 0.078 (8)	0.470 ± 0.092 (7)
Pituitaries	1.756 ± 0.256 (8)	2.375 ± 0.695 (10)	0.834 ± 0.180* (10)

For this and following table and figures: mean ± standard error (number of rats).
*$p < 0.05$ from estrus and tumor.

ing rats did not modify GnRH receptor levels in this tissue (Table 1).

Pituitaries from luteoma bearing rats and control estrous rats showed a similar number of GnRH receptors, determined by saturation analysis. In contrast, when luteoma bearing rats were treated with buserelin for a month, a significant decrease ($p < 0.05$) in the pituitary GnRH receptor number was observed, indicating downregulation of these receptors (Table 1). Receptor levels were approximately five times more abundant in the hypophysis than in ovarian tissues.

Effect of Buserelin on Steroid Hormone Secretion in Luteoma and Estrous Ovaries: Organ Incubations

When ovaries or hemitumors were incubated in vitro, a significant difference in basal E_2 and progesterone release into the medium was observed. Tumors secreted higher amounts of estradiol and lower amounts of progesterone than the ovaries in estrus used as controls (Fig. 3, upper and middle panels) ($p < 0.05$). This difference was observed both after 1.5 and 3 h of incubation (data from 3 h incubation not shown). No significant differences were observed in the basal testosterone levels between both tissues (Fig. 3, lower panel).

When buserelin ($1 \times 10^{-7}M$) was added into the medium, no significant differences in hormone levels were observed for any of the three hormones tested either in the luteoma or in the ovaries (Fig. 3, upper, middle, and lower panels), indicating that this compound had no effect on basal secretion.

Effect of Buserelin on Steroid Hormone Secretion in Luteoma and Superovulated Ovaries: Cell Cultures

A significant difference in basal progesterone and estradiol levels was observed in the luteal cell cultures between tumor and control cells (Table 2). The former secreted significantly higher levels of estradiol and lower titers of progesterone than the controls, in agreement with the current results in organ incubation experiments. These high levels of estradiol production in luteoma cells imply an increase in aromastase activity, which is tested when

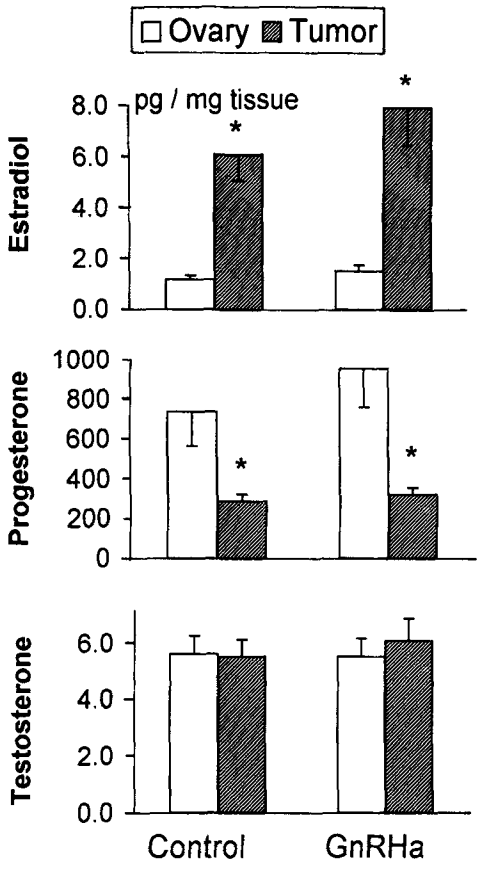

Fig. 3. Steroid hormones in culture medium (pg/mg tissue) after 1.5 h of organ cultures. Effect of GnRHa (buserelin: $1 \times 10^{-7} M$). *$p < 0.05$. In each group of ovaries (with or without GnRH-a) $n = 18$. In each group of tumors (with or without GnRH-a) $n = 24$.

Table 2
Basal Steroids Secretion (pg/μg DNA) in Culture Medium of Luteal Cells

	Control	Tumor
Progesterone	67.7 ± 6.8 (9)	48.9 ± 4.4* (11)
Estradiol	2.2 ± 0.1 (6)	7.7 ± 1.0* (12)

*$p < 0.05$.

androstendione is added to the culture medium. In tumor cells, buserelin did not modify basal progesterone production after 4 h of incubation, but significantly blunted the LH-induced progesterone increase (Fig. 4, lower panel) ($p < 0.01$). In luteal cells from superovulated prepubertal ovaries, buserelin did not modify basal progesterone secretion. After LH stimulation, an insignificant decrease in progesterone output was observed (Fig. 4, upper panel), marking another difference between both tissues.

With regard to estradiol, neither LH nor buserelin significantly altered its secretion in either kind of luteal cells after 7 h of incubation, implying that aromatase activity

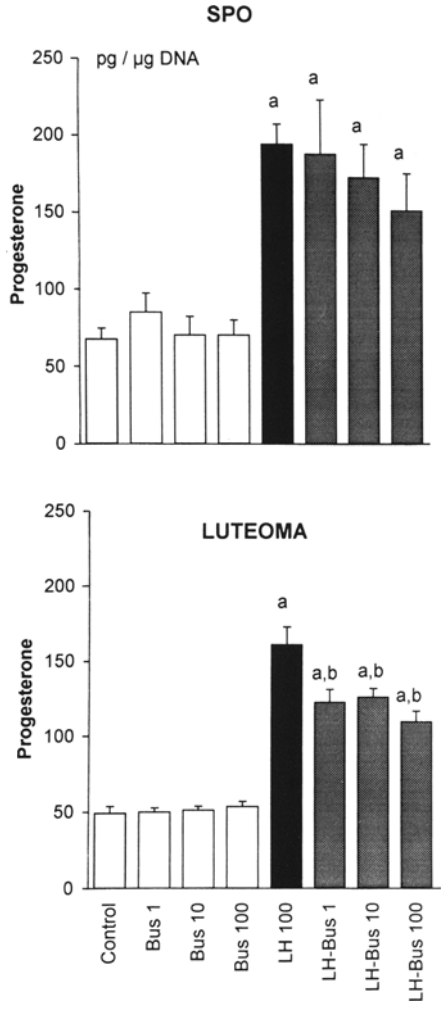

Fig. 4. Progesterone in culture medium (pg/μg DNA) after 4 h of cell incubations. For this and the following figure: SPO: cells from superovulated prepubertal ovaries. LH and Bus (buserelin) in ng/mL. Cell culture experiments were repeated three times. Number of wells varied from 6 to 12 in each experimental group. a: $p < 0.05$ from control, Bus 1, Bus 10, and Bus 100. b: $p < 0.05$ from LH.

does not seem to be modified by either stimuli present in the culture medium (Fig. 5, upper and lower panels).

Discussion

Similarities and differences were observed between control ovarian and tumoral tissues. With regard to GnRH receptors, they are present in luteoma tissue and no differences in maximal binding and Kd were observed when compared to estrous ovaries *(11)*. It has been described that GnRH binding sites in ovarian tissue are not influenced by the stage of the estrous cycle *(12)*. Furthermore, in agreement with previous works *(11,29)*, GnRH receptors in ovarian tissue (control or luteoma) was lower than that found in the anterior pituitaries of these animals. However, a different response to buserelin treatment between pituitaries and tumoral tissues was observed. Whereas the GnRH analog decreased the maximal binding in the pituitary, no effect

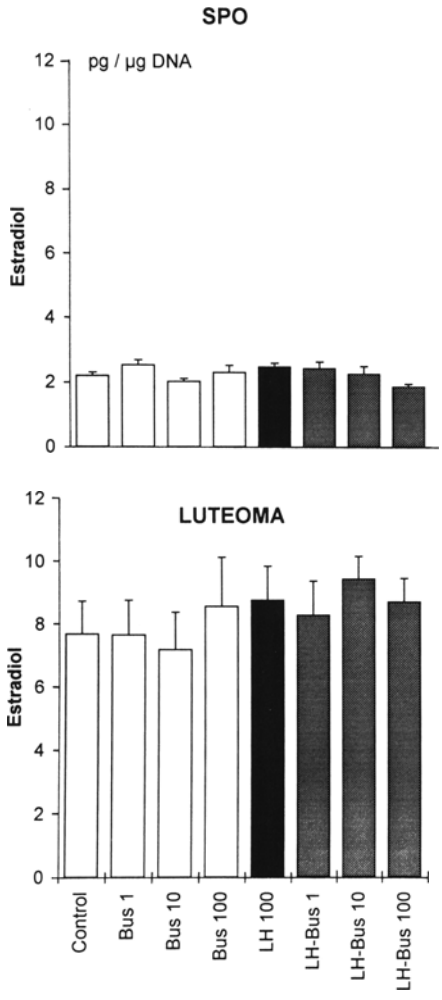

Fig. 5. Estradiol in culture medium (pg/µg DNA) after 7 h of cell incubations.

was observed in the luteoma, indicating that these receptors are not downregulated by constant stimulation, in contrast to what was described in the pituitary *(30)*. A lack of desensitizing response or even an upregulation of GnRH receptors by in vivo administration of the decapeptide was also found in the normal ovary *(12,31)*. All these data taken together suggest that GnRH receptors are not strongly modified in this tumoral tissue. Furthermore, as they are not desensitized, they could play a role in the inhibition of tumor growth observed in vivo with buserelin treatment in luteoma bearing rats in a previous work *(6)*. GnRH receptors have also been detected in epithelial carcinoma and stromal tumors of the human ovary *(32)*. These observations render this experimental luteoma an excellent model for the investigation of GnRH action in its antitumoral effect.

To further investigate the effects of GnRH or its analogs on this tumoral tissue, the steroidogenic response to buserelin was evaluated both in basal and LH-stimulated conditions. Significant differences in hormone levels were observed when comparing tumor alto normal tissue in organ incubations. Luteoma secreted 417% more estradiol and 61% less progesterone than estrous ovaries. These results suggest that steroid synthetic pathways may be altered in the tumoral tissue, especially taking into consideration that estrous ovaries secrete very low progesterone levels *(33,34)*. Buserelin in vitro did not modify progesterone, estradiol, or testosterone secretion either in tumors or estrous ovaries. The lack of response of basal steroid secretion to GnRH analogs in normal ovarian tissue has been described previously *(22,35)*.

A second in vitro model, monolayer cultures of control and luteoma cells, was undertaken to evaluate if GnRH analogs modified gonadotrophin-stimulated progesterone and estradiol secretion. Once again, significant differences in basal steroid secretion were detected. Progesterone levels were 28% lower and estradiol 250% higher in luteoma than in control cells. In this case, estradiol levels are a reflection of aromatase activity, since androstenedione was added to the culture medium. These differences, though less marked than in organ incubations, where the histology of the tissue is conserved, reinforce the idea of steroid synthetic alterations in luteoma cells, including an activation of aromatase activity.

In human ovarian tumors, different patterns of steroid hormones secretion have been described according to their histological type. Whereas serum estradiol and progesterone levels are higher in women bearing epithelial non-endocrine tumors than in normal postmenopausal women, only progesterone is increased over the levels observed in the follicular phase of the menstrual cycle *(36–40)*. In contrast, in far less frequently appearing endocrine tumors as, for example, a juvenile granulosa cell tumor *(41)* or an adult granulosa cell tumor *(42)*, very high levels of estradiol similar or higher than those of the midcycle peak accompanied by relatively low progesterone levels have been described. The secretory pattern of our experimental luteoma, which is also of endocrine nature, resembles this latter kind of tumor, indicating that similar alterations of the synthetic pathways may be occurring.

With regard to the effect of buserelin on steroid secretion in the cell cultures, this analog did not modify basal progesterone levels, but significantly inhibited LH-induced progesterone secretion in luteoma cells, as was observed in previous preliminary results *(6)*. It has been proposed that the GnRH-induced decrease in progesterone production may result from the inhibition of progesterone biosynthesis and/or the stimulation of its breakdown *(43, 44)*. In cells from superovulated prepubertal rats, although LH induced a similar increase in progesterone levels, buserelin inhibition did not reach statistical significance. These results in control cells differ from those described by Clayton et al. *(11)* or Behrman et al. *(22)*, who showed that buserelin inhibited LH- or hCG-induced progesterone secretion after short incubation periods. Both those experiments were done with higher concentrations of the GnRH analog, which may account for the differences observed. In the case of similar

results described by Labrie et al. *(45)*, though similar drug concentrations were used, experimental conditions differed, since their experiments were performed 2–3 d after plating. Nevertheless, it is interesting to note that the low GnRH analog concentrations used in this work marked a difference in sensitivity of the progesterone inhibiting effect of buserelin between both cell kinds. With regard to estradiol, no variations in estradiol levels were observed either in luteoma or control cells with any of the stimuli added after 7 h of incubation. Previous works described a stimulatory effect of LH on estradiol secretion, but at a much lower concentration (1 ng/mL) and after a longer incubation time (24 h) *(46)*. The lack of effect of buserelin on estradiol secretion in cell cultures is in agreement with the authors' results in organ incubation experiments, though an inhibiting effect on basal levels was described in human granulosa-luteinic cells *(23)*. Furthermore, in rat luteinized granulosa cells, GnRH inhibited LH-stimulated estradiol secretion *(47)*.

In summary, these ovarian tumors possess GnRH receptors in similar number to normal ovarian tissue and they are not downregulated by constant GnRH analog administration. In addition, a clear difference in basal steroid secretion between tumoral and normal tissue was described, being that of the tumors similar to the secretory patterns of some types of human endocrine ovary tumors. Furthermore, a differential sensitivity to the GnRH analog in its progesterone inhibiting effect was observed. This suggests that the inhibition in tumor growth observed in vivo with buserelin may not only be a result of pituitary GnRH receptor desensitization and gonadotrophin suppression, but also be a consequence of direct action of the GnRH analog on the tumoral tissue.

Materials and Methods

Adult female virgin Sprague-Dawley rats from the Instituto de Biología y Medicina Experimental colony were housed in groups in an air-conditioned room, with lights on from 0700 to 1900 h. They were given free access to laboratory chow and tap water.

For the in vitro organ incubations, cell culture and GnRH receptor experiments, animals were bilaterally ovariectomized and one ovary was implanted into the spleen 1 mo before the experiments, as previously described *(4,6)*. For GnRH receptors, one group of ovarian grafted animals was also administered a pellet of buserelin, a GnRH agonist (Buserelin Implant, a gift from Hoechst Buenos Aires, 0.33 mg/rat) on the same day of the ovarian graft.

Two sources of luteal tissue were selected as controls. Ovaries from estrous rats, with fresh corpora lutea, were used as controls for organ incubation and GnRH receptor experiments.

For histological comparative analysis and cell culture experiments, luteinized ovaries from 23–25 d-old rats, injected with 25 UI PMSG (Endocorion, Elea, Buenos Aires) and 25 UI hCG (Novormon, Syntex, Buenos Aires) 48 h later were used 5 d after hCG injection, as they provide a highly luteinized tissue.

GnRH Receptors

Preparation of $^{[125]}$Iodine GnRH Analog

[D-Ser(-tBu)^6-des-Gly^{10}]-GnRH-*N*-ethylamide (GnRH-a) (gift from Hoechst, Buenos Aires) was used as tracer and unlabeled hormone in the binding assay *(48)*. GnRH-a was iodinated using a chloramine-T method. Briefly, 2 μg of GnRH-a were iodinated in the presence of 2 mCi $^{[125]}$iodine (New England, MA) and 0.2 μg chloramine-T. Reaction proceeded for 2 min. The procedure was repeated once, and reaction was stopped by transferring to a carboxy-methyl cellulose column and eluting unbound iodine with 0.002*M* ammonium acetate, and the labeled analog with 0.060*M* ammonium acetate. The iodinated analog was stored at 4°C in this last buffer and used within 3 wk of preparation. The specific activity of each preparation was assessed by self-displacement in the receptor assay using a crude membrane fraction prepared from pooled pituitaries. Specific activities ranged from 400 to 800 μCi/μg. Maximum binding of the trace, determined by incubation with excess pituitary membranes, was 40–65%. Affinity constants and binding capacities were corrected for maximum binding of the trace.

Assay of Pituitary and Ovarian GnRH Receptors

Rats were killed by decapitation and tissues were quickly removed and stored at –70°C. Pituitaries were thawed and homogenized in assay buffer (Tris-HCl 10 m*M*, 0.1% bovine serum albumin (Sigma, St. Louis, MO), and 1 m*M* dithiothreitol (Sigma), pH 7.6 at 4°C) in a glass homogenizer. Homogenates were prepared immediately before addition to assay tubes. Ovaries and luteoma were homogenized in an ultraturrax and centrifuged at 400*g* for 5 min; the supernatant was then centrifuged at 11,500*g* for 15 min. The pellet was resuspended in 1 mL assay buffer and filtered through nytex. For saturation analysis, the homogenate from a single pituitary, ovary or luteoma was used to prepare five incubation mixtures (three for total binding and two for nonspecific binding). Each tube had approx 1 mg tissue (for pituitaries) or 2 mg tissue (for ovarian tissues) in 300 μL buffer. Homogenates were incubated with 5–8 × 10^4 cpm [^{125}I] GnRH-a in a total volume of 500 μL assay buffer. Ligand concentrations were near saturating, representing about 85% receptor occupancy. Nonspecific binding was determined by addition of 1 × 10^{-6}*M* of unlabeled GnRH-a, and represented 5–8% of total iodinated tracer.

For Scatchard analysis, 300 μL ovarian or tumor homogenates (pool of tissue from 2–3 rats in each group diluted to a concentration of 2 mg/300 μL) were incubated in the presence of increasing concentrations of labeled analog (5000–120,000 cpm), in a total volume of 500 μL Parallel

incubation mixtures contained $2 \times 10^{-7}M$ unlabeled GnRH-a to assess nonspecific binding. Experiments for Scatchard analysis were repeated three times.

In all cases, tubes were incubated for 120 min on ice, and reaction was terminated by centrifugation at 16,000*g* for 20 min at 4°C. The supernatant was discarded, and the pellets were counted in a γ-spectrometer (efficiency 73%).

Organ Incubation Experiments

Rats bearing ovarian implants and rats in estrus were decapitated and the luteoma and ovaries were, respectively, removed and weighed. As tumors were larger than ordinary ovaries, each one of them was halved in order to have approximately the same weight per incubation vial, and thus a similar degree of diffusion of drugs into the tissue. Control ovaries were incubated one whole ovary per vial. Tumors and ovaries were placed in individual vials containing 4 mL of Medium 199 (Sigma), HEPES 25 m*M* (Sigma), and BSA 0.1% (Sigma). The vials were placed in a Dubnoff metabolic shaker at 37°C, 5% CO_2/95% O_2, for a 30-min preincubation period. The medium was discarded and the tissues were washed with 1 mL fresh medium that was also discarded. Fresh medium (4 mL) without (control) or with buserelin $1 \times 10^{-7}M$ was added to each vial, and the glands were incubated for 3 h. Sample aliquots were taken at 1.5 and 3 h. When taking the 1.5 h sample, volume was restored (with or without buserelin $1 \times 10^{-7}M$, respectively). In control tissue (estrous ovaries), one ovary from each rat was used, one as control and the other one was stimulated with buserelin; in the case of luteoma, one half was used as control and the other was incubated in the presence of the GnRH-a. Samples were stored at –20°C until assayed for estradiol, progesterone, and testosterone by RIA. Results were expressed as pg of hormone/mg of tissue.

Tumor Cell Cultures

Animals were operated on as described to induce the development of the luteoma and were left undisturbed for 1 mo. Cells from ovarian tumors, as well as from 23–25 d-old superovulated rats, were isolated after collagenase digestion for in vitro cultures *(6,23,46)*. Plastic 24 multiwells were coated with rat tail collagen. Approximately 500,000 cells were seeded per well in 1 mL of media (DMEM-F12 with 2,2 g/L sodium bicarbonate and 0.1% BSA). Stimuli, LH (100 ng/mL in each well), GnRH-a (Buserelin: 1, 10, or 100 ng/mL in each well) or the combination of both drugs, were added immediately after plating. Wells containing only medium were used as controls. Plates were placed in a culture chamber (5% CO_2 in air at 37°C). After 4 h of incubation, two aliquots of 180 μL were taken from each well and frozen for progesterone measurements. Androstenedione, as substrate for estrogen biosynthesis, was added in a final well concentration of 0.25 μ*M* and cells were further incubated for 3 h *(46)*. Thereafter, two 300-μL aliquots were taken from each well and frozen for estradiol determinations. Tumor cell cultures were repeated three times. Number of wells varied from 6 to 12 in each experimental group. Results are expressed as pg/μg DNA of hormone levels in each well. DNA content of each well was measured at the end of the experiment—as described in ref. *49*.

Histological Analysis

For light microscopic examination, the ovaries from superovulated rats and 1–2 mo old luteoma (*see* tissue donors in Materials and Methods) were fixed in 10% buffered formalin, embedded in paraffin wax, and sectioned at 4 μm using a microtome. Sections were stained with H&E using standard procedures. Several slides were examined from each tumor and the correspondent controls. Both tissues show a high degree of luteinization, but, unlike the normal prepubertal stimulated ovary (Fig. 1, left panel), no follicles were observed in the tumor slides examined (Fig. 1, right panel). The spleen, into which it the original ovary was grafted, can also be observed (Fig. 1, right panel) (final magnification: ×40).

RIAs

Estradiol, progesterone, and testosterone in culture media were estimated by RIA using specific antisera kindly provided by Dr. G. D. Niswender. Labeled hormones were purchased from Amersham. Assay sensitivity for estradiol was 1.7 pg and intra- and interassay coefficients of variation were 9.3 and 11.4%, respectively. Assay sensitivity for progesterone was 50 pg and intra- and interassay coefficients of variation were 7.5 and 11.9%, respectively. Assay sensitivity for testosterone was 12.5 pg and intra- and interassay coefficients of variation were 7.8 and 12.3%, respectively.

Statistical Analysis

Differences in means of hormonal levels in cell culture experiments were analyzed using the one way analysis of variance followed by Tuckey's test. Student's *t*-test and Student's paired *t*-test were used to analyze hormonal levels in organ incubation experiments.

Scatchard analysis of binding data was performed by a computer curve-fitting program (Ligand) for a single class of binding sites. Changes in receptor number for a single treatment were analyzed using oneway analysis of variance followed by Tuckey's test. In all cases, $p < 0.05$ was considered significant.

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