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Biphasic Regulation of the Gonadotropin-Releasing Hormone Receptor by Receptor Microaggregation and Intracellular Ca^{2+} Levels

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SUMMARY

In the present work we show that Ca^{2+} is both necessary and sufficient to evoke homologous up-regulation of the gonadotropin-releasing hormone (GnRH) receptor. Extracellular Ca^{2+} as well as RNA and protein synthesis were required for this event, and it was blocked by Ca^{2+} ion channel blockers. Drugs which stimulated increased intracellular Ca^{2+} levels also stimulated receptor up-regulation and enhanced responsiveness even in the absence of added GnRH. Such drugs were effective below the concentrations needed to evoke luteinizing hormone (LH) release, suggesting that enhanced levels of Ca^{2+} ion, rather than LH depletion, is the responsible agent. A GnRH antagonist did not evoke up- or down-regulation; however, a conjugate of this antagonist, which stimulated microaggregation of the GnRH receptor, also stimulated these biphasic actions. In contrast to up-regulation, down-regulation of the GnRH receptor appears to be Ca^{2+}-independent and does not require RNA or protein synthesis. These data are consistent with a model in which microaggregation of the GnRH receptor is the final step in common to a branched pathway consisting of Ca^{2+}-dependent (LH release, enhanced sensitivity, up-regulation) and Ca^{2+}-independent (desensitization, down-regulation) events.

INTRODUCTION

In addition to stimulation of pituitary gonadotropin release, GnRH[1] provokes a biphasic response of receptor numbers and cell responsiveness (1–8). Initially (0–3 hr), receptor numbers and response to GnRH are diminished; at later times (5–10 hr), receptor numbers and cell sensitivity to GnRH are increased.

Ca^{2+} clearly fulfills the requirements of a second messenger for GnRH-stimulated LH release (9), however, it does not mediate the action of GnRH on desensitization (10). It can be shown, for example, that removal of extracellular Ca^{2+} blocks GnRH-stimulated LH release, while desensitization occurs to the same extent as when extracellular Ca^{2+} is present. Similarly, the Ca^{2+} ionophore A23187 can evoke LH release but not desensitization. Interestingly, a bivalent conjugate of a GnRH-receptor antagonist behaves as an agonist, stimulating both release *and* desensitization as a result of its ability to provoke GnRH receptor-receptor microaggregation (11), apparently the final step common to both processes.

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[1] The abbreviations used are: GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′N′*-tetraacetic acid; M199/BSA, medium 199 containing 0.3% bovine serum albumin; CMC, carboxymethyl cellulose; D600, methoxyverapamil.

In the present study we have examined the role of Ca^{2+} in GnRH-stimulated increased receptor number and cell sensitivity in an effort to integrate this process into the steps already characterized as significant in the mechanism of action of this hormone.

METHODS

Preparation and culture of dispersed rat pituitary cells. Pituitary dispersion was performed as described before (12). Briefly stated, whole pituitaries (about 50–100) were removed from female weanling rats and collected into sterile Hepes-buffered (10 mM) medium 199 (M. A. Bioproducts) containing 0.3% BSA (M199/BSA, Sigma Fraction V) at room temperature. When collection was completed, the medium was decanted and replaced with M199/BSA containing gentamycin (20 μg/ml) (Schering). The pituitaries were exposed to this solution for 15–20 min at room temperature.

The pituitaries were finely minced in a Petri dish containing 5 ml of M199/BSA, then transferred to a 50-ml centrifugation tube containing M199/BSA and permitted to settle. The medium was decanted and replaced twice to remove the remnants of lysed cells disrupted during mincing of the tissue.

A 10-ml portion of freshly prepared solution containing 0.25% collagenase (Worthington CLS II, 126 units/mg), and 0.1% hyaluronidase (Sigma) in M199/BSA was sterilized by filtration through 0.45-μm membranes (Millipore HAWP) and added to the tissue fragments. The centrifuge tube was capped, placed on its side in a 37° water bath, and shaken at 100 cycles/min. At 3-min intervals, the tissue suspension was gently passed five times through a 10-ml sterile disposable pipette containing a cotton plug at the suction end.

The solution was filtered through organza cloth to remove residual tissue fragments, and the filtrate was brought to final volume with 50

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ml of M199/BSA. After centrifugation at 125 × *g* for 10 min, the pellet was resuspended in 1–2 volumes (milliliters per pituitary) of M199/BSA containing 10% horse serum and 2.5% fetal calf serum (M. A. Bioproducts). One-milliliter aliquots of the cell suspension were placed in 2.2-ml wells (MultiWell tissue culture places; Costar) and maintained for 2 days at 37°. After this period, the plate was inverted and the cells were washed twice in 2-ml portions of M199/BSA (to remove sera and unattached cells) and covered with 1.0 ml of the same medium containing selected concentrations of GnRH (National Pituitary Agency) or other drugs (A23187, Calbiochem; D-*p*-Glu^1-D-Phe^2-D-Trp^3-D-Lys^6-GnRH, Dr. John Stewart, University of Colorado; D600, Knoll Pharmaceutical; veratridine, cycloheximide, and EGTA, Sigma Chemical Company). After 3 hr or the indicated time, the media were removed from the cells by aspiration and assayed for LH by radioimmunoassay.

Radioimmunoassay. The radioimmunoassay for LH was performed as recommended in the instructions of the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases (NIADDK) kit, with antiserum LH S-6, LH I-5 for iodination, and RP1 LH standard, provided by the NIADDK Hormone Distribution Office and the National Pituitary Agency. Bound and free hormone were separated with immobilized protein A (13). Inter- and intra-assay variances were typically 10% and 7%, respectively.

Radioligand preparation. A superactive, degradation-resistant analogue of GnRH, D-Ser(*t*-Bu)^6-des-Gly^{10}-ethylamide (Buserelin, from Mr. V. Wagner, Hoescht-Roussel Pharmaceuticals, Inc.), was iodinated using chloramine T and purified by CMC column chromatography, as described previously (14). Maximal bindability of the radioligand was assessed by binding with excess plasma membrane receptor. The membrane fraction used was prepared from weanling rats, and maximal bindability was about 50%. Specific activity (assessed by self-displacement assay) was 850–1250 μCi/μg for different preparations. For antibody-stripping studies, AB-9113 was used. This antibody does not bind (<0.01%) GnRH analogues with the des^{10}-Pro^9-ethylamide substitution.

Binding assay. The binding assay was conducted in polypropylene Microfuge tubes which were precoated overnight with 1% BSA. Cells were scraped from culture plates after the culture medium was replaced by a solution of 3 mM EDTA containing 5 mM Tris-HCl/0.1% BSA (pH 7.4), then washed twice in the assay buffer (10 mM Tris-HCl, pH 7.4). The assay, run in triplicate, contained approximately 60,000 cpm of [^{125}I]Buserelin and 1–2 × 10^6 cells, in the presence or absence of 10 μM GnRH, and was conducted for 2 hr in an ice bath. No additional binding is seen when the time of incubation is increased. The final assay volume was 200 μl. After this period, the mixture was poured onto a Whatman GF/C filter (soaked overnight in 0.1% BSA) then washed twice with ice-cold 2.5-ml volumes of the incubation buffer. Radioactivity was determined by *gamma* spectroscopy. Specific binding was determined by subtracting nonspecific binding (in the presence of excess unlabeled GnRH) from total binding (no GnRH). Scatchard analysis of the binding data, using linear regression analysis to establish best-line fit, was used to determine the number and binding affinity of GnRH receptors.

General. The "conjugate" of the GnRH antagonist was prepared as described previously (15) from the reaction product of ethylene glycol bis-succinimyl succinate with D-*p*-Glu^1-D-Phe^2-D-Trp^3-D-Lys^6-GnRH (i.e., antagonist dimer) coupled to (cross-reactive) antibody No. 5111. This "conjugate" stimulates LH release from pituitary cultures (16) and cell desensitization (11) in a manner consistent with receptor microaggregation (17).

For normalization of cell numbers, cells were quantified by measurement of DNA (18) in samples prepared and treated in a manner identical with that employed for the samples assayed for responses, or in the experimental cells themselves. Typically, agreement was better than 5% between wells. All data were derived as the mean ± standard error of the mean of values obtained in three or six replicates. In order to pool data from several experiments, the data were expressed as percentage of control cells which had a mean number of receptors = 3.86 ± 0.32 fmoles/10^6 cells.

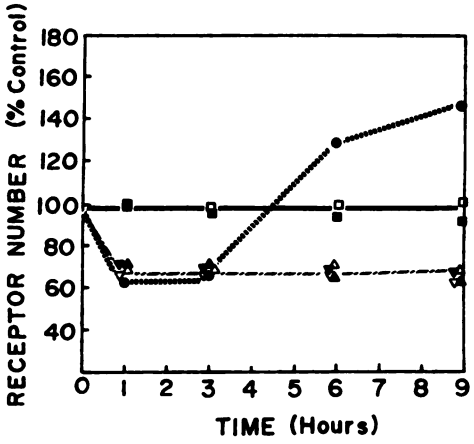

FIG. 1. *Effect of incubation with GnRH on receptor number*

Two-day pituitary cultures were incubated for the indicated time period with medium alone (□) or medium containing 3 mM EGTA (■), 1 nM GnRH (●), 3 mM EGTA + 1 nM GnRH (△), 0.1 mM D600 + 1 nM GnRH (▼), actinomycin D (1 μg/ml) + 1 nM GnRH (▲), or cycloheximide (1 μg/ml) + 1 nM GnRH (▽). After this time period receptor numbers were determined as described under Methods. Values for standard error of the mean (N = 3–6) were omitted for clarity and were generally 10%.

RESULTS

When pituitary cell cultures were incubated with 1 nM GnRH for the continuous period shown (Fig. 1), the receptor number underwent biphasic regulation. At initial times (0–3 hr), receptor numbers decreased below control levels, then recovered (3–5 hr) and overshot the control values (6–9 hr). The initial decrease in receptor number is not likely due to occupancy of the GnRH receptor by the homologous hormone, since the cells are washed under conditions which would be expected to elute the GnRH [$t_{1/2}$ for dissociation is a few minutes (9)] and the 2-hr binding assay provides sufficient time for exchange of any remaining GnRH with the higher-affinity radioligand. In studies in which antisera AB-9113 (which binds GnRH, but not the radioligand) was included during the binding incubation (titer 1:100), no difference was seen in numbers of receptors as compared with measurements made in the presence of preimmune serum.

Up-regulation did not appear to require continual presence of GnRH since exposure for 20, 30, or 360 min resulted in similar levels of receptor during the up-regulated phase (Table 1). Likewise, inclusion of AB-9113 (titer 1:100) after the initial exposure did not inhibit up-regulation. In order to monitor the efficacy of the washing procedure employed, LH release was also measured. Washing of the cells is sufficient to arrest this event, suggesting that GnRH can be removed.

Levels of receptors in control incubations (incubated in either medium alone or medium containing 3 mM EGTA) did not change during the study (0–9 hr), and recovery, but not down-regulation, was blocked by 3 mM EGTA or 0.1 mM D600 (which blocks entry of extracellular Ca^{2+}). Similarly, up-regulation (but not down-regulation) appears to require macromolecular synthesis, since it is blocked by 1 μg/ml of either actinomycin D or cycloheximide. These concentrations of inhibitors nei-

TABLE 1

Effect of time of exposure to 1 nM GnRH on receptor number at 9 hr compared with control cells (medium only)

Cells were incubated for the indicated time with GnRH. Extracellular LH was determined by radioimmunoassay (Period I). The cells were then washed and incubated with or without GnRH AB9113 (titer 1:100) for the remaining time to 9 hr. At that time, receptor numbers were determined as described under Methods and released LH was measured by radioimmunoassay (Period II).

Treatment	Receptor no. relative to control values	LH release relative to control values	
		Period I	Period II
Control	100%	100%	100%
2 min	98%	102%	97%
20 min	138%	113%	99%
20 min (then GnRH Ab)	139%	111%	97%
60 min	139%	217%	102%
60 min (then GnRH Ab)	142%	202%	103%
360 min	145%	608%	100%
540 min	143%	652%	—

ther stimulated LH release nor inhibited cell responsiveness to GnRH (Table 2).

Both up- and down-regulation appeared to require occupancy by an agonist; antagonist occupancy did not evoke either process (Fig. 2), and, indeed, blocked the ability of GnRH to evoke this biphasic regulation. Interestingly, when the ability to cross-link (i.e., microaggregate) receptors was conferred upon the antagonist, it became able to stimulate both up- and down-regulation.

Agents which elevate intracellular Ca^{2+} levels (ionophore A23187, veratridine; Fig. 3) evoked up-regulation (in a Ca^{2+}-dependent fashion) without the characteristic

TABLE 2

LH release in response to exposure of pituitary cell cultures to the indicated treatment

Two-day pituitary cultures were incubated with the indicated compounds. After 3 hr, media were collected and assayed for LH by radioimmunoassay using National Institutes of Health preparation RP1 as a standard. The values shown are means (N = 3–6), the standard errors of the mean being less than 12%.

Treatment	LH release
	ng/100 ng DNA/3 hr
No addition	3.8
GnRH	
1 μM	42.2
1 nM	22.4
1 nM + cycloheximide (1 μg/ml)	21.2
1 nM + actinomycin D (1 μg/ml)	23.4
Veratridine	
0.1 mM	38.7
1 μM	4.2
A23187	
10 μM	44.2
100 nM	3.2
10 nM	4.3

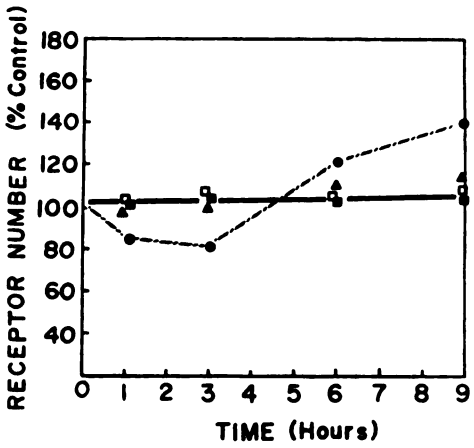

FIG. 2. *Effect of incubation with GnRH antagonist on receptor number*

Two-day pituitary cultures were incubated for the indicated time period with both 1 nM GnRH + 5 μM GnRH antagonist (▲), 1 nM GnRH antagonist (■), 5 μM GnRH antagonist (□), or conjugate (●) prepared as described under Methods. The conjugate concentration was sufficient to evoke 50% LH release in 3 hr as compared with GnRH. Receptor numbers were determined as described under Methods. Values for standard error of the mean (N = 3–6) were omitted for clarity and were generally 10%.

down-regulation seen in response to GnRH. Interestingly, these drugs were active at concentrations which did not stimulate measurable LH release above basal levels (Table 2).

DISCUSSION

Consistent with another recent report (7), GnRH occupancy of its receptor promotes an initial decrease then an increase in receptor numbers but not affinity ($3.0 \pm 0.6 \times 10^9$ M^{-1}). Occupancy of the receptor by an antagonist is not in itself sufficient to evoke down- or up-

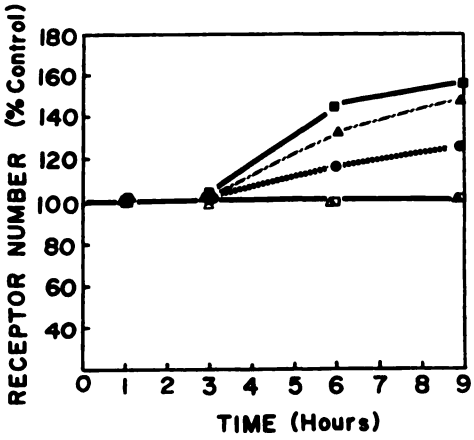

FIG. 3. *Effect of agents that stimulate increased intracellular Ca^{2+} levels on receptor number*

Two-day pituitary cultures were incubated for the indicated time period with 1 μM veratridine (■), 100 nM (▲) or 10 nM (●) A23187, 1 μM veratridine + 3 mM EGTA (▽), or 100 nM A23187 + 3 mM EGTA (△). After this period, receptor numbers were determined as described under Methods. Values for standard error of the mean (N = 3–6) were omitted for clarity and were generally 10%.

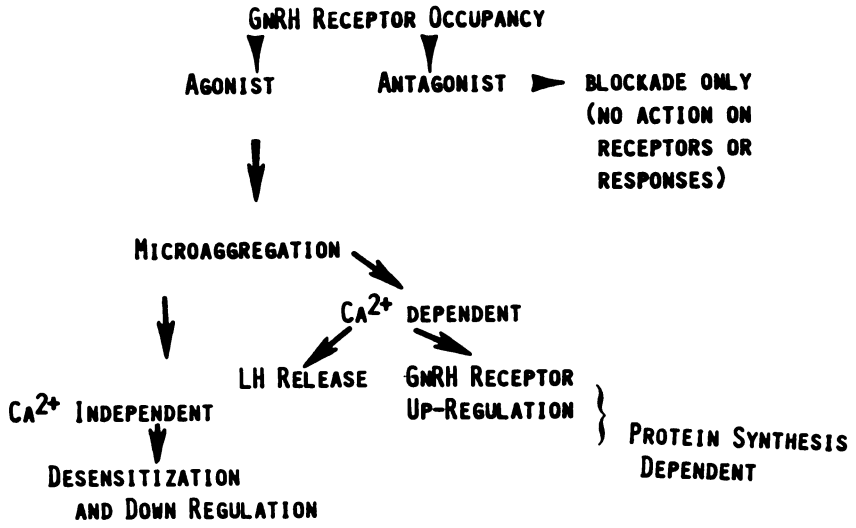

FIG. 4. *Theoretical scheme integrating experimental observations on GnRH-mediated actions*
The evidence supports a model in which receptor microaggregation is the final step in common to GnRH-mediated actions. This leads to Ca^{2+}-independent and Ca^{2+}-dependent pathways. The Ca^{2+}-dependent pathway includes a biosynthesic-dependent component.

regulation and blocks these actions of GnRH. Up-regulation, but not down-regulation, can be blocked by depletion of extracellular Ca^{2+} or the presence of the Ca^{2+} ion channel blocker D600 (methoxyverapamil). Similarly, we have shown previously that desensitization (which can be measured as rapidly as 1 hr after a 20-min exposure to GnRH) is a Ca^{2+}-independent process [not blocked by low extracellular Ca^{2+} or by D600 (10)].

Additional evidence that up-regulation is a Ca^{2+}-mediated process comes from the observation that ionophore A23187 and veratridine (which mobilize extracellular Ca^{2+} to an intracellular site by acting at loci other than the GnRH receptor) both mimic the actions of GnRH and provoke increases in GnRH receptor number without the initial drop in receptor numbers seen in response to the releasing hormone. It is also evident that Ca^{2+} and ionophores have no specific action on the recognition of GnRH by its receptor (19). In addition, the enhancement of receptor number appears to be independent of LH release, since this action persists [unlike release (20)] when releasing hormone is washed out. Moreover, low concentrations of both A23187 and veratridine were capable of stimulating up-regulation while LH release was not evoked (21, 22). At higher concentrations of ionophore a smaller increase in receptors was noted, suggesting a biphasic action of Ca^{2+}. A regulatory role for Ca^{2+} in gene expression (such as may be the case in the present study) is consistent with another report (23) implicating such an action at low concentrations (ED_{50} about 100 μM).

The observation that up-regulation is uncoupled from LH release makes unlikely the possibility that up-regulation is mediated by receptors which may be on secretion granules (24).

Additionally, unlike desensitization, up-regulation appears to be dependent on both protein and RNA synthesis, as low concentrations of cycloheximide and actinomycin D block the latter process.

Both down- and up-regulation are provoked by receptor microaggregation, since a GnRH antagonist, which alone provokes neither process, becomes active when the ability to dimerize receptors is conferred upon it. It appears likely that such actions are mediated by the ability of this conjugate to cross-link GnRH receptors and mimic GnRH actions.

While it is attractive to consider that a relationship exists between receptor number and cell responsiveness, the precise relationships remain to be established, some workers arguing for such a relation (5, 25, 26) and others arguing against one (6, 7). The present study suggests that during the period of receptor recovery (5–10 hr), when the cells are clearly refractory to GnRH (7), receptor number and cell responses are clearly uncoupled. Following short-term exposure, when the effect of LH depletion is minimized, down-regulation and desensitization appear to have some components in common.

Thus, the present data support a model shown in Fig. 4 in which GnRH-receptor microaggregation is the last step in common to a branched pathway. This event evokes four physiological actions attributed to the releasing hormone: stimulation of LH release, receptor down-regulation, desensitization (11), and receptor up-regulation. Down-regulation and desensitization, on one hand, appear to be Ca^{2+}-independent whereas gonadotropin release and GnRH receptor up-regulation are Ca^{2+}-mediated actions.

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Note added in proof. We have recently become aware that the actions of GnRH on receptor up-regulation can be mimicked by cyclic AMP and its analogues (R. N. Clayton, personal communication).

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