Homologous Regulation of Gonadotropin-Releasing Hormone Receptors in Cultured Pituitary Cells

Abstract. *Specific receptors for gonadotropin-releasing hormone (GnRH) in cultured rat pituitary cells were increased by subnanomolar concentrations of GnRH agonists and decreased by high concentrations of these peptides. The antagonist [D-Phe², Pro³, D-Phe⁶]GnRH did not alter GnRH binding capacity and blocked the increase in sites induced by GnRH. These findings provide direct evidence for the homologous regulation of GnRH receptors by physiological concentrations of the hypothalamic peptide, an action that could mediate the cyclical and postcastration increases in GnRH receptors and responsiveness of the pituitary gonadotrophs.*

The hypothalamic control of gonadotropin secretion is mediated by the decapeptide gonadotropin-releasing hormone (GnRH), which stimulates the secretion of luteinizing hormone (LH) and follicle-stimulating hormone after binding to high-affinity receptors on the gonadotroph plasma membrane. Receptor sites for GnRH have been characterized in pituitary membrane preparations (*1*) and in dispersed pituitary cells (*2*). Recently, significant variations in pituitary GnRH receptor content have been observed during the rat estrous cycle (*3–5*) and lactation (*5, 6*), and after castration in male and female rats (*5–8*). The increase in pituitary GnRH receptors after gonadectomy can be prevented by steroid replacement, by administration of a GnRH antagonist or GnRH antibodies, and by lesions of the median eminence (*5–8*). These findings suggest that endogenous GnRH has a role in the regulation of its own receptor sites in vivo, a concept supported by the ability of GnRH and a potent GnRH agonist to increase the number of pituitary GnRH receptors in adult male rats (*3, 8*) and in ovariectomized ewes (*9*). In this report, we describe the regulatory effects of GnRH on its own receptor sites in cultured rat pituitary cells.

For this study we used cells from anterior pituitary glands of adult female rats. The cells were dispersed as described (*2*) by a modification of the method of Nakane *et al.* (*10*) and cultured for 48 hours. The pituitary cells were then exposed to selected concentrations of GnRH or a superactive analog (*11*) by addition of 20 μl of peptide solution to each well. Six to 9 hours later, the cells were removed from the plates for assay of GnRH receptor sites (*7*). The binding capacity of dispersed rat pituitary cells for GnRH after 2 days in culture was 1.96 ± 0.16 fmole per 10^6 cells (± standard deviation). If one assumes that gonadotrophs comprise 8 percent of the pituitary cell preparation (*12*), this is equivalent to about 15,250 ± 1,250 binding sites per cultured gonadotroph. Exposure of such cell preparations to native GnRH caused significant changes in the GnRH binding capacity. Concentrations of the decapeptide between $10^{-11}M$ and $10^{-8}M$ consistently increased the total binding capacity (Fig. 1), with a maximum increment of 60 percent at GnRH centrations between $10^{-10}M$ and $10^{-8}M$. The higher binding observed in GnRH-treated cells was due to an increase in the number of binding sites, with no change in binding affinity, as shown by Scatchard analysis (Fig. 2). This phenomenon of GnRH up-regulation was not only dose-related but also time-dependent. Thus, a significant effect was observed as soon as 6 hours after addition of the peptide, and was maintained for up to 15 hours.

Higher concentrations of GnRH ($10^{-6}M$ and above) reduced the total binding capacity of the cell preparation by up to 55 percent of the control value. The GnRH agonist D-Ser⁶-EA (*11*), which has a high binding affinity and is resistant to degradation, was also able to regulate the GnRH binding capacity (Fig. 1). The maximum up-regulation was obtained with concentrations of this agonist around $10^{-11}M$, and loss or down-regulation of GnRH receptors was observed with agonist concentrations of $10^{-9}M$ and above.

In contrast, equivalent concentrations (10^{-6} and $5 \times 10^{-6}M$) of the GnRH antagonist [D-Phe², Pro³, D-Phe⁶]GnRH did not reduce the binding capacity for the radioiodinated D-Ala⁶ analog (Fig. 3). It should be noted that the association constant (K_a) of this antagonist measured by pituitary radioligand-receptor assay is 4.2×10^8 per mole, similar to that of native GnRH (3.0×10^8 per mole). The up-regulatory effect of $10^{-9}M$ GnRH was abolished by the simultaneous addition of $10^{-6}M$ antagonist to the cell cultures, with reduction of the receptor increase from 71 to 3 percent of the control value.

These data demonstrate that GnRH can modify the number of its own pituitary receptors in vitro. The bioactive GnRH concentration in the culture dish falls progressively because of the rapid degradation of the peptide, which has a half-life of about 80 minutes in the cell incubation system (Fig. 4). The cells are thus exposed to GnRH in a single pulse-like manner, and under these conditions the regulatory effect of GnRH is both time- and dose-dependent.

A significant increase in binding ca-

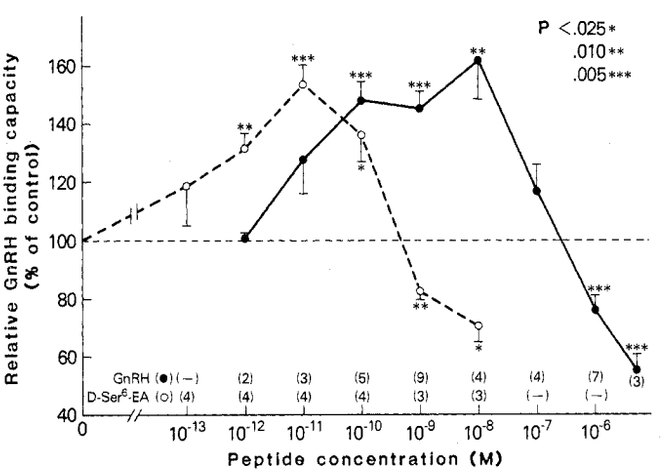

Fig. 1. Concentration-dependence of the regulatory effects of GnRH and D-Ser⁶-EA on GnRH binding capacity of cultured rat pituitary cells (day 2). After 48 hours in culture, the cells were exposed to selected concentrations of GnRH or analog by addition of 20 μl of peptide solution to each well. The cells were removed from the plates 6 to 9 hours later by gentle scraping with a rubber policeman. The culture dishes were rinsed with Medium 199 and the cells from six wells (6×10^6) were pooled and centrifuged at 24°C for 10 minutes at 100*g*. The pellet was washed twice by resuspension in 10 ml of calcium-free Medium 199, then resuspended in 630 μl of assay buffer (10 m*M* tris-HCl, *p*H 7.7, containing 1 m*M* dithiothreitol and 0.1 percent bovine serum albumin). The radioreceptor assay was performed at 0°C in 12 by 75 mm borosilicate tubes: 100 μl of cell preparation (~ 10^6 cells), 100 μl of either assay buffer or a solution of D-Ala⁶-EA ($3 \times 10^{-6}M$), and 100 μl of tracer (^{125}I-labeled D-Ala⁶-EA; 60,000 count/min, or ~30 fmole per tube, specific activity 1100 to 1300 μCi/μg). Incubations were terminated after 120 minutes by dilution with 4 ml of ice-cold phosphate-buffered saline, *p*H 7.4, followed by immediate filtration through glass-fiber filters (Whatman GF/C) (*2*). After several washings the filters were analyzed by gamma spectrometry to determine the tissue-bound radioactive tracer. In all experiments, each assay point included three tubes for total binding and three for nonspecific binding. The results were expressed as the percentage of the total binding of the control cell preparation.

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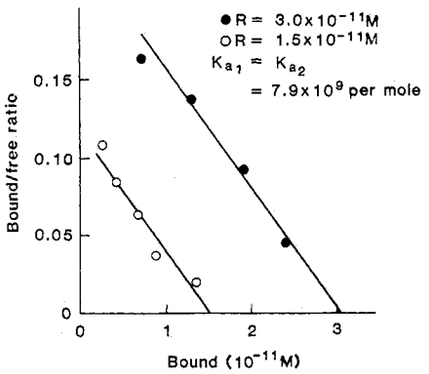

Fig. 2. Scatchard plots of GnRH receptor binding in pituitary cells. Day 2 cells were collected 8 hours after addition of GnRH to a final concentration of $10^{-9}M$ (●). At the same times, untreated control cells were collected and assayed (○). Scatchard analysis of receptor concentration (R) and association constant (K_a) was performed on data derived from saturation studies with increasing concentrations of ^{125}I-labeled D-Ala^6-EA, a computer program being used for resolution of the Scatchard plots into one or two binding sites (*1*).

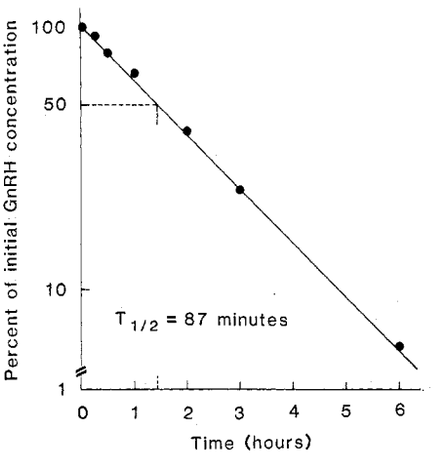

Fig. 4. Degradation of native GnRH during incubation with cultured pituitary cells in Medium 199 containing 10 percent horse serum. After addition of $3 \times 10^{-8}M$ GnRH to 2-day cultured cells (10^6 cells per 35-mm well), portions of medium were removed at selected times, centrifuged at 100g for 10 minutes at 4°C, and analyzed in a pituitary radioligand-receptor assay for GnRH as previously described (*7*). In other experiments not shown, the rapid degradation of GnRH was found to be predominately due to the presence of horse serum in the culture medium.

pacity was observed as soon as 6 hours after exposure of pituitary cells in vitro to GnRH agonists. In contrast, the increase in GnRH receptors in castrated male rats was detected at 18 hours (*6*), and the accompanying increase in plasma LH was observed at 12 to 15 hours. The lag period between the effect of endogenous GnRH on LH release, and on its own receptor regulation, is of the order of 3 to 6 hours, a time that is compatible with our observations in vitro. Also, estrogen treatment of castrated animals restores pituitary GnRH binding capacity to the precastration level in only 3 hours (*5*).

Concentrations of GnRH between $10^{-11}M$ and $10^{-7}M$ induced up-regulation of GnRH receptors, with a maximum effect between $10^{-10}M$ and $10^{-8}M$; up-regulation also occurred with around $10^{-11}M$ concentrations of the D-Ser^6-EA agonist. These concentrations are of the same order of magnitude as those necessary to induce 50 percent of the maximum LH release in the same cell system, $2.3 \times 10^{-10}M$ and $4 \times 10^{-12}M$, respectively, for GnRH and the D-Ser^6-EA analog. A similar ratio between these two peptides was observed in the apparent binding affinity measured in dispersed pituitary cells at 37°C (*13*). Taken together, these observations indicate the physiological relevance of the present data with isolated pituitary cells. In addition, immunoreactive GnRH concentrations in blood from the rat pituitary stalk have been reported to be between $10^{-12}M$ and $10^{-10}M$ during metestrus, diestrus, and early proestrus, and to increase to $10^{-9}M$ around the time of the LH surge (*14*). Such endogenous GnRH concentrations are compatible with a role of this peptide in the maintenance of pituitary GnRH receptor sites and their increase at these stages of the estrous cycle. However, this does not appear to be sufficient to explain the rapid decrease in receptor number that occurs at the late proestrous stage by a simple down-regulation. The increased GnRH concentrations in blood from the pituitary stalk after castration (*15*) are consistent with the up-regulatory concentration range of the endogenous peptide.

The ability of the GnRH antagonist to block the increase in pituitary receptors induced by native GnRH further indicates the receptor-mediated nature of the GnRH effect. Also, the inability of the antagonist to influence GnRH receptors at saturating concentrations (up to $10^{-6}M$) indicates that not only occupancy but also activation of the GnRH sites is necessary to produce the regulatory effect on pituitary receptor concentration. This is consistent with the generally recognized inability of antagonist ligands to induce receptor regulation and refractoriness, as first demonstrated for the β-adrenergic receptor (*16*). The precise mechanism by which GnRH agonists alter the concentration of their membrane receptors is still unclear. One possibility is that the increase in receptor sites is related to stimulation of gonadotropin secretion, with insertion of additional receptors upon fusion of the secretory granules with the plasma membrane (*17*). However, in other experiments we have observed a dissociation between the processes of receptor regulation and gonadotropin secretion, indicating that synthesis or unmasking of new receptors is more likely to account for the increase in GnRH binding sites.

These findings demonstrate the importance of GnRH as an endogenous physiological regulator of its own receptor sites in the pituitary gland. This hypothalamic peptide may not be the only factor responsible for such regulation, since the direct effect of a steroid has been shown by the ability of the androgen 5α-dihydrotestosterone to reduce GnRH binding sites in cultured rat pituitary cells (*18*). Furthermore, estrogen causes a twofold increase in GnRH receptors in the monkey pituitary (*19*), an action that could be mediated by increased hypothalamic secretion of GnRH. Thus the integrated effects of pituitary GnRH secretion and gonadal steroid feedback are probably

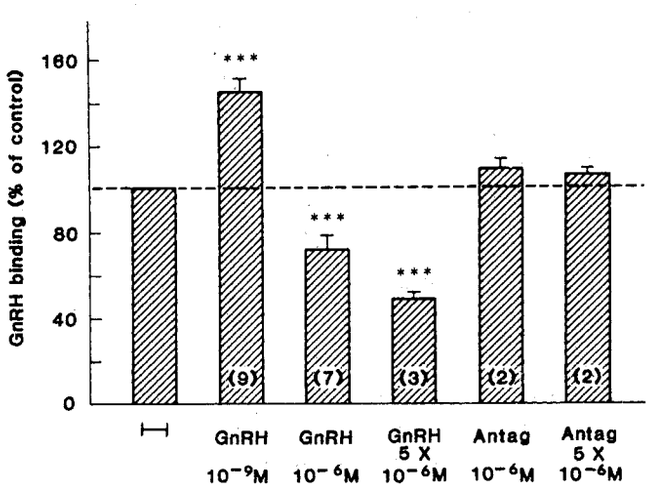

Fig. 3. Regulatory effect of native GnRH on its own receptors in cultured rat pituitary cells. Relative binding capacity is computed as the percentage of the control preparation for each experiment. The GnRH antagonist [D-Phe^2,Pro^3,D-Phe^6]-GnRH did not show any regulatory effect at these concentrations. ***, $P < .005$.

responsible for the changing profile of pituitary GnRH receptors and responsiveness during physiological variations in reproductive function.

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αA-Crystallin Messenger RNA of the Mouse Lens: More Noncoding Than Coding Sequences

Abstract. *The 14S messenger RNA (1300 to 1500 nucleotides) for the αA chain of α-crystallin of the mammalian lens is nearly three times larger than required to code for the polypeptide that contains 173 amino acids. As a means of accounting for this anomaly, a complementary DNA clone for the mouse αA-crystallin messenger RNA was constructed in pBR322 and sequenced. Derivation of the protein sequence from the nucleic acid sequence showed that mouse αA-crystallin is similar to that of other organisms. The messenger RNA contains 536 nucleotides located on the 3′ side of the coding region, excluding the polyadenylate stretch. This 3′ sequence does not encode any other crystallin and has multiple termination codons in the three possible reading frames.*

The ocular lens of vertebrates synthesizes and accumulates large amounts of structural proteins called crystallins (*1*). The crystallins contribute 90 percent of the soluble lens protein and are highly conserved during evolution (*2*). There are four immunologically separate classes of crystallins called α-, β-, γ- and δ-crystallin. δ-Crystallin occurs only in birds and reptiles (*3*). The messenger RNA's (mRNA) for the δ-crystallins (*4*), the αB chain of α-crystallin, the β-crystallins, and the γ-crystallins (*5, 6*) are, like those of most eukaryotic mRNA's, only slightly larger than their coding requirements. By contrast, the mRNA for the αA chain of α-crystallin from calves (*7*) and rats (*8*) has a sedimentation value of 14*S* (1300 to 1500 nucleotides), which is nearly three times the size necessary to code for the 173 amino acids of this polypeptide (*9*). The 14*S* αA-crystallin mRNA is capped (*10*) and polyadenylated (*11*). The evolutionary conservation of the large size of the αA-crystallin mRNA suggests a critical function outside of the region coding for αA-crystallin. It has been suggested that the 14*S* mRNA may be bicistronic and encode two α-type crystallin polypeptides (*12*). To gain insight into the function of the sequences of the 14*S* αA-crystallin mRNA, we have constructed and sequenced a complementary DNA (cDNA) clone for this mRNA from the mouse lens.

Polyadenylated mRNA from 1000 lenses of 5- to 10-day-old mice (NIH general-purpose stock) was extracted, subjected to reverse transcription, made double-stranded with DNA polymerase I of *Escherichia coli*, and inserted into the Pst I site of the plasmid pBR322 by the G-C (guanine-cytosine) procedure (*13*). The recombinant plasmids were used to transform *E. coli* LE392. More than 1000 recombinant cDNA clones were obtained. A putative αA-crystallin cDNA clone was identified by the technique of positive hybrid-selection (*14*), with a rabbit reticulocyte lysate (New England Nuclear) as the cell-free protein synthesizing system. A recombinant plasmid (pMαACr2) selectively hybridized mRNA from the mouse lens that directed the synthesis of αA crystallin in vitro (data not shown). Nucleotide sequence of the pMαACr2 insert was determined by the method of Maxam and Gilbert (*15*) (Fig. 1). The sequence and translation of the cloned cDNA in pMαACr2 are shown in Fig. 2a. Comparison with the amino acid sequence for the αA chain of α-crystallin of other mammals (*16*) revealed that pMαACr2 contains nearly a complete copy of the codons for this polypeptide. Nucleotides 1 to 490 (Fig. 2a) predict an amino acid sequence identical to amino acids 10 to 173 of rat αA-crystallin (*16*). This result is consistent with the finding that the evolution of α-crystallin is very slow (*2*). It has been estimated from protein sequencing studies that amino acid substitutions in α-crystallin occur at a rate of approximately 1 percent per 17 million years (*2*). Since rats and mice are believed to have diverged about 10 million years ago (*17*), we would not expect more than a single amino acid difference in the αA-crystallin polypeptides of these rodents. The nucleotide sequence of pMαACr2 also reveals an unusually long sequence 3′ to the coding region. The polyadenylation signal AATAAA (A, adenine; T, thymine), which is present in all eukaryotic mRNA's (*18*), is present in the 3′ region starting at position 1002 in Fig. 2a.

We examined all three possible reading frames of the cloned insert in pMαACr2 for initiation and termination codons to test the possibility that another protein may be encoded within this cDNA. The results demonstrated that multiple termination signals exist in the nucleotide sequence 3′ to the αA-crystallin coding region, regardless of the reading frame considered (Fig. 2b). Only reading frame 1 lacks a termination codon within the known coding region. Considering the location of possible initiation and termination codons on the 3′ side of the coding region, the largest polypeptide that could be synthesized with any reading frame is 60 amino acids (reading frame 3 in Fig. 2b).

A possible criticism of this stop codon analysis is its sensitivity to single nucleotide errors in the sequence determination. An added or deleted base shifts the termination codon into a new reading frame. To investigate the possibility that nucleotide sequences coding for crystallin polypeptides are contained in the cloned cDNA in pMαACr2 we used a dot matrix computer analysis that de-

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