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Regulation of Gonadotropin Release, GnRH Receptors, and Gonadotrope Responsiveness: A Role for GnRH Receptor Microaggregation

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Gonadotropin-releasing hormone (GnRH) stimulates luteinizing hormone (LH) and follicle-stimulating hormone (FSH) release from pituitary gonadotrope cells. Additional receptor-mediated actions of the releasing hormone include homologous regulation of both the GnRH receptor and of cell responsiveness. While it is apparent that the release mechanism is Ca^{2+} mediated, it remains unclear how this receptor-mediated action is integrated with regulation of the receptor and with cell responsiveness. It is the purpose of this review to describe the requirements for gonadotropin release as well as for receptor and response regulation in order to prepare an integrated model for these actions of the releasing hormone.

BINDING OF GnRH AND ITS ANALOGS BY THE RECEPTOR

Biochemistry

The binding step has been studied in great detail owing to the availability of a wide variety of useful analogs. Highly satisfactory radioligands can be prepared by using high-affinity, metabolically stable agonists [1,2]. Such synthetic compounds have in common the presence of a D-amino acid^6 (inhibiting degradation) and the substitution des-Gly^{10}-Pro^9 ethylamide (enhancing receptor binding affinity when combined with the D-amino acid^6). Detailed studies employing these analogs (which can be radioiodinated to high specific activity) have shown changes in GnRH receptor number (but not binding affinity) during the rat estrus cycle [3–5], lactation, castration, and aging [3] and other endocrine states. In a general way, the frequency of the receptors is predictive of the responsiveness of the gonadotrope cell to GnRH.

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Agonist occupancy of the plasma membrane GnRH receptor [6] mobilizes extracellular Ca^{2+} via a plasma membrane Ca^{2+} ion channel. The channel appears to be similar to that found in nervous and muscle tissue. Interestingly, differences in structure-activity relationships with Ca^{2+} ion channel antagonists have revealed that the channel is not identical to that observed in these other tissues [7].

Molecular Biology

Observations describing the molecular biology of the receptor can be made by preparation of fluorescent GnRH analogs which can be monitored on living cells by image-intensified microscopy [8,9]. As has been observed for many polypeptide hormones, the fluorescently labeled GnRH (presumably occupying the receptor, since the process is saturable and specific for gonadotropes) can be seen to undergo patching, capping, and internalization at 37°C.

Recently [10], a metabolically stable gonadotropin-releasing hormone agonist (D-Lys^6-GnRH) was coupled to electron-opaque markers (colloidal gold and ferritin) in order to characterize the intracellular pathway of the releasing hormone bound by pituitary gonadotropes. This approach has the advantage of increasing the resolution of localization to a "circle of uncertainty" about 10–20-fold smaller than that which can be obtained by autoradiography. After an initial uniform distribution on the cell surface, the derivatives were taken up individually as well as in small clusters in coated and uncoated membrane invaginations and moved to the lysosomal compartment either directly or after passage through the Golgi apparatus. The results suggest that labeled GnRH or GnRH-receptor complex may be routed to two distinct intracellular compartments: the lysosome and the Golgi cisternae.

An early question therefore was, is patching, capping and internalization necessary for the molecular events which ensue? In order to answer this question, D-Lys^6-GnRH (which has a reactive amino group) was covalently attached to an immobile support [11,12]; LH release could then be measured when GnRH was prevented from entering the cell. The derivative provoked LH release at full efficacy and therefore suggested that internalization is not necessary for GnRH to exert its effect.

It was apparent that vinblastin could inhibit receptor patching, capping, and internalization in response to the releasing hormone but could not inhibit LH release [11]. This also suggested that the process of patching, capping, and internalization could be uncoupled from release. Patching and capping refer to events that can be seen by image-intensified microscopy. The resolution of such a technique is only about a hundred molecules. Therefore events which occur as the result of receptor dimerization or multimerization (that is receptor *micro*aggregation, which is described below) would not be seen by this technique.

An additional approach has been a two-incubation experiment [11]. In these studies, cells were first incubated in various concentrations of GnRH for various times. After about 15 min at ED_{50} or higher concentrations, considerable internalization of the releasing hormone occurs. If the releasing hormone is then removed from outside the cells, one of two things will happen. If the internalized GnRH is sufficient to support continued gonadotropin release, this event should continue. If, in contrast, a continuously applied extracellular source of GnRH is required, then the response system should undergo extinction—the latter appeared to be the case.

After washing GnRH from outside the cells, the cells rapidly stop releasing gonadotropin. Extinction occurs. Consequently, an externally applied, continuous source of GnRH is necessary for the response system to continue. It then appeared that patching, capping, and internalization were not necessary for the releasing hormone to exert its effect.

Receptor-Receptor Interactions: Microaggregation

In order to examine the significance of receptor-receptor interaction at levels below that which can be measured by image intensification, additional use can be made of the GnRH analogs. Because of the interest of drug companies in this compound and support from the Contraceptive Development Branch of the NIH, a large number of GnRH antagonists are presently available. Many of these antagonists appear to work by the classic pharmacologic means; that is, they occupy the receptor without efficacy (ie, no gonadotropin release). A particular GnRH antagonist was used: D-pGlu^1-D-Phe^2-D-Trp^3-D-Lys^6-GnRH [13]. The substitution of D-amino acids in the first three positions leads to marked antagonism intrinsic in this molecule. It has no measurable agonist activity. The substitution with a D-Lys^6 at the sixth position provides protection against biologic degradation and, in addition, introduces the only amino group in this molecule (the N-terminus is blocked, pyro-Glu^1). It was then a simple matter to prepare a GnRH antagonist dimer with a very short bridge length (about 12 Å) between the antagonist molecules (Fig. 1). This could then be used almost like a male-male plumbing fitting to change the specificity of an antibody initially directed against the antagonist. It is possible then to prepare a molecule which is a derivatized antibody having a GnRH antagonist dimer at either F_{ab} arm. This compound, when applied to cells, has considerable efficacy as an agonist. This strange event, ie, the conversion of a GnRH antagonist to an agonist as a result of its divalency, was a confusing result. In a number of human disease states, antibodies have been identified which cross-link receptors and consequently provide agonist

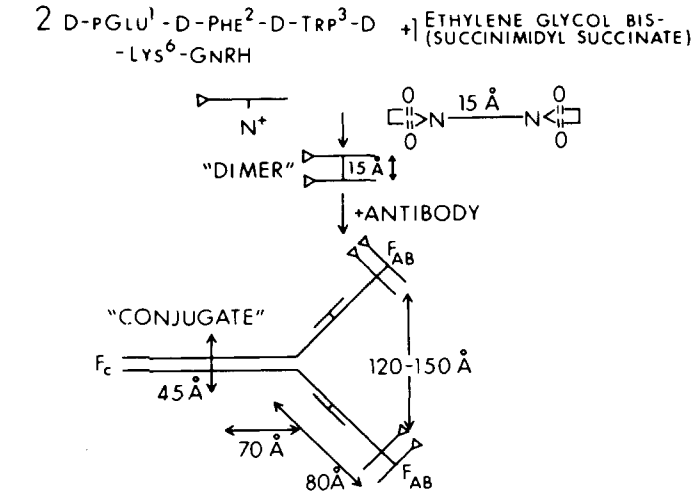

Fig. 1. Schematic drawing showing synthesis of antibody conjugate. Two GnRH antagonist molecules are cross-linked by (lysyl) epsilon aminos. Further incubation with a cross-reactive antibody results in antagonists being separated by 120–150 Å. The precise spatial arrangement of the dimer as bound to the antibody has not been determined and should not be presumed from the graphic.

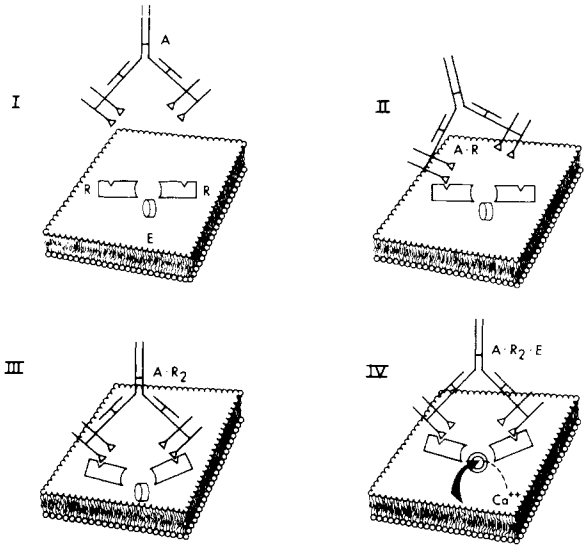

Fig. 2. Schematic model showing presumed cross-linking (*micro*aggregation) of GnRH receptors by antibody conjugate described in Figure 1 and text. The precise spatial arrangement of the dimer as bound to the antibody has not been determined and should not be presumed from the graphic.

efficacy. Because of these observations, receptor-receptor interactions were considered in the present situation.

Indeed, when a papain or reduced-pepsin cleavage product of the antibody (ie, univalent "antibody") is coupled with the dimer, we produce pure antagonist. The antibody alone has no agonist efficacy and consequently the inescapable conclusion appeared to be that receptor-receptor interactions, that is, the dimerization (or higher order interactions) of receptors, could stimulate the response system. A model for what might be occurring is presented in Figure 2. An antagonist then might be a compound which could occupy the receptor, but, because of its inability to promote receptor-receptor dimerization, would then behave antagonistically. When one takes an antagonist and confers upon it the ability to cross-link receptors, we are now able to see agonist efficacy (ie, LH release). Interestingly, the dimer alone (ie, no antibody) appeared to be too short to cross-link receptors and it remained an antagonist. It was also possible to demonstrate that the efficacy of the agonist in this system shared much in common with the authentic native molecule of GnRH. Both, for example, are inhibited by calmodulin antagonists. Both, additionally, require extracellular calcium. It was therefore presumed that the mechanism by which the receptor dimerization event was able to provide agonist efficacy was very much similar to that which was provided by the native molecule (that is, GnRH). It was also possible to use this technique to potentiate the action of a GnRH agonist (D-Lys^6-GnRH) [14]. This compound is a biologically stable compound because of the D-amino acid^6 substitution, and, additionally, has the amino group for substitution to prepare dimers. Following preparation of the agonist dimer, it was possible to show that when it was administered to cells at a ED_{10} dose, its efficacy could be superpotentiated by addition of antibody, suggesting, then, that the agonist was able to occupy receptors and then at the appropriate concentrations was able to be cross-linked by antibodies to that molecule.

Recently, computer simulations [15] were prepared for this model. If we assume that two receptors are able to come together about a previously closed calcium ion channel and if these two receptors are able to stimulate opening of the ion channel, an equilibrium model can be built. Such a model, interestingly, fits the data within approximately 5% over five dose logs.

RECEPTOR-MEDIATED ACTIONS OF THE RELEASING HORMONE

Gonadotropin Release

While Ca^{2+} clearly fulfills the requirements of a second messenger [dealt with previously:16,17], and is tightly coupled to gonadotropin release [17a], the steps which follow its mobilization remain unclear. A likely candidate for the intracellular calcium receptor is calmodulin, which redistributes inside pituitary cells following treatment with GnRH. Gonadotropin-releasing hormone provokes calmodulin disappearance in the cytosolic fraction and appearance in the plasma membrane fraction [18]. The constitutive expression of calmodulin in these cells suggests that redistribution may actually reflect translocation within the cells. A recent study [18a] has revealed that calmodulin appears to localize in the GnRH-receptor patch [8,9] following activation of the cells with the releasing hormone.

A related observation is that calmodulin inhibitors [19], some of which are highly specific [20], block GnRH-stimulated LH release in the same potency order as they bind calmodulin. Thus, a role for calmodulin in this system appears reasonable. Although we are uncertain of the action of calmodulin once occupied with calcium, a number of possibilities have been described previously [18].

Desensitization

In addition to LH release, receptor occupancy (by an agonist) leads to desensitization (that is, refractoriness of the cells as a result of prior administration of GnRH, [21]). This process has some fundamental differences with the release process. It can be shown, for example, that desensitization, unlike the gonadotropin-releasing process, is not calcium dependent. Advantage was taken of a technique for growing pituitary cells on beads [22]. This provides a good model system for kinetic studies, as exposure to GnRH for short periods can be accomplished. It was possible to demonstrate conditions which led to desensitization following a physiological dose of GnRH. The question remained, then, whether this reduced efficacy was a result of LH depletion from the cells or whether it was a true receptor-mediated sort of desensitization (ie, receptor depleted population). In order to answer this question in a very direct manner, responsiveness of the cells following administration of ionophore A23187 was measured. As mentioned above, calcium behaves as a second messenger in this system; therefore, ionophore A23187, which allows calcium to freely enter the cell, behaves as a secretagogue [22]. It was possible to show then that if A23187 was first given to cells, then washed out and GnRH given in the second administration, that the cells *did* have the potential to respond fully to this challenge, suggesting that LH depletion was not the explanation for this reduced sensitivity. This also suggested that secretion and desensitization may be mediated by fundamentally different processes in this system.

In order to probe this question further, advantage was taken of the fact that extracellular calcium is an absolute requisite for GnRH-stimulated LH response from these cells [23]. In these studies, calcium was first removed from outside the cells, GnRH was then added, and the receptor was occupied under a condition (diminished extracellular calcium) which did not lead to gonadotropin release. Here we have a condition in which the receptor is occupied but gonadotropin is not released from the cells because of the low extracellular calcium. The GnRH was then removed and calcium added back. Surprisingly, we found cells so treated to be desensitized. Thus, a result of occupancy is desensitization whether or not release of LH occurs. This suggests, in addition, that the release system and the desensitization system are mediated by fundamentally different means. It further could be seen that, while LH release has an absolute requirement for calcium, desensitization appears not to be a calcium-mediated event. It could also be seen that GnRH antagonist alone did not lead to desensitization. Thus, simple receptor occupancy did not result in desensitization. Agonist occupancy of the receptor is required for desensitization to occur.

It was, at this point, desirable to see if the dimerized antagonist could provide desensitization. Indeed, it was able to do so [24]; suggesting then that there is a slightly more complicated and branched mechanism of response of this system, which will be described below.

Biphasic Regulation of the Receptor

Pituitary cell cultures were used to examine the effect of GnRH and other treatments on the GnRH receptor [25]. The GnRH occupancy of its receptor promotes an initial decrease, then 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-regulation and blocks these actions of GnRH. Up-regulation, but not down-regulation, can be blocked by depletion of extracellular Ca^{2+} or by the presence of the Ca^{2+} ion channel blocker D600 (methoxyverapamil).

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+} by acting at loci other than the GnRH receptor, both stimulate LH release and provoke increases in GnRH receptor number without the initial drop in receptor numbers seen in response to the releasing hormone. Indeed, the enhancement of receptor number appears to be independent of LH release since this action persists [unlike release, 22] 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 [25,26]. 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 is consistent with another report [27] 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 up-regulation is mediated by receptors which may be on secretion granules. 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

TABLE I. Requirements of Gonadotropin Release, Receptor Regulation, and Regulation of Gonadotrope Responses*

	LH release	Desensitization and down-regulation	Up-regulation
Evoked by			
Antagonist	No	No	No
Agonist	Yes	Yes	Yes
Microaggregation	Yes	Yes	Yes
Extracellullar Ca^{2+}	Yes	No	Yes
Requires:			
Time	0–3 hr	0–3 hr	5–10 hr
Protein synthesis	No	No	Yes
Extracellular Ca^{2+}	Yes	No	Yes

*While desensitization and down-regulation share much in common, this Table should not be taken to suggest that they are conclusively manifestations of the same process.

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. The requirement of gonadotropin release, receptor regulation, and regulation of cell responsiveness is shown below in Table I.

While it is attractive to consider that a relationship exists between receptor number and cell responsiveness the precise relationships remains to be established, some workers arguing for such a relation [29–31] and others arguing against one [32,33]. The present studies suggest that during the period of receptor recovery (5–10 hr), when the cells are clearly refractory to GnRH [33], receptor number and cell responses are clearly uncoupled. Following short-term exposure, when the effect of LH depletion is minimized, down-regulation and desensitization clearly appear to have some components in common.

Domains Associated With the GnRH Receptor

While it has not yet been shown that GnRH itself stimulates receptor microaggregation as a component of its mechanism of action, the observation that a GnRH antagonist can be converted to an agonist (as described above) suggests that it may be convenient to consider that there are two functional domains associated with the GnRH molecule. One of these is required for recognition of the molecule by the active site of the receptor ("R" site), and the other is necessary for activation of the effector, likely though microaggregation ("M" site). An agonist possesses both sites. An antagonist in this scheme possesses an R site, (thus binding somewhat similarly to an agonist, which appears to be the case [34]) but not an M site. It becomes an agonist when it is (artificially) conferred with the ability to crosslink receptors. While a compound lacking both sites would not be either a receptor agonist or an antagonist, one could imagine that compounds with M sites but no R sites could be biologically significant. Such compounds might lack specificity but could activate the system by provoking microaggregation. Compounds which restrict the movement of the GnRH receptor to a small domain (and thus might enhance the chances of random microaggregation) are an example of a compound of this type. Positively charged high molecular weight polymers may be an example of such a compound [34a]. A related type of compound might have a specificity component conferred upon it by recognition of a site other than the active site (ie, that which recognizes the GnRH molecule).

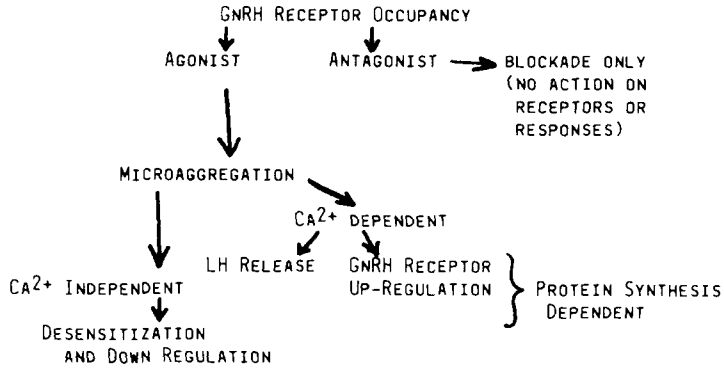

Fig. 3. Integrated model showing roles of receptor occupancy and microaggregation, Ca^{2+}, and synthetic events in regulation of receptors, LH release, and cell responsiveness.

An example of this type of molecule would be an antibody developed against the GnRH receptor. Such specific anti-receptor antibodies clearly stimulate other hormone receptors.

Considerable evidence exists which suggests that the R and M sites correspond to definite physical domains of the GnRH molecule: pGlu^1-His^2-Trp^3-Ser^4-Tyr^5-Gly^6-Leu^7-Arg^8-Pro^9-Gly^{10}-NH_2. It was initially observed that deletion of the His^2 (ie, des-His^2-GnRH) resulted in a molecule which bound to the GnRH receptor (albeit with very low affinity) but which had no LH-releasing activity. This molecule first demonstrated the potential of synthesizing GnRH analogs which behaved as antagonists.

Further studies [35] identified the His^2 and, later, Trp^3 as sites which could be substituted without total loss of receptor-binding activity but with loss of the ability to evoke LH release (receptor level competitive antagonists). Substitutions in this position (His^2-Trp^3) then allow the molecule to be recognized by the receptor (R site) but not activate the effector (likely M site). Thus the His^2-Trp^3 region likely corresponds to the M site.

Conformational analysis of the GnRH molecule suggests that the least energy state favors close association of the N- and C-termini (perhaps something like the letter C). Deletion of pGlu^1 or even opening the pyro-Glu ring results in dramatic loss of binding affinity. Substitutions at the Gly^{10} position such as replacement with an ethylamide group results, when coupled with a D-amino acid in the sixth position, in considerably enhanced receptor binding affinity. Interestingly, except for this "substituted 10" derivative, peptides with less than ten amino acids have not been identified which bind with appreciable affinity. This observation may emphasize the importance of the one to ten amino acids in receptor recognition; this site is likely the R site.

CONCLUSIONS: A UNIFIED MODEL

The available data support a model shown in Figure 3 in which GnRH-receptor microaggregation is the last step in common to a branched pathway. This event evokes at least four physiological actions attributed to the releasing hormone: LH

release, receptor down-regulation, desensitization, and receptor up-regulation. Down-regulation and desensitization, on the one hand, appear to be Ca^{2+} independent, while gonadotropin release and GnRH receptor up-regulation are Ca^{2+}-mediated actions.

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