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MOLECULAR MECHANISM OF GONADOTROPIN RELEASING HORMONE ACTION*

P. MICHAEL CONN, DAPHNE STALEY, HYDER JINNAH and MICHAEL BATES
Department of Pharmacology, University of Iowa, College of Medicine, Iowa City, IA 52242, U.S.A.

Summary—This work summarizes some of our studies related to the mechanism of action of gonadotropin releasing hormone including those leading to identification of the three steps of the gonadotropin releasing process: receptor binding, mobilization of extracellular calcium, and granule exocytosis. Evidence is also presented to suggest how these steps are integrated one to another and how they are integrated to other actions of the releasing hormone such as regulation of target cell sensitivity, and receptor regulation.

INTRODUCTION

Gonadotropin releasing hormone (GnRH) stimulates luteinizing hormone (LH) and follicle stimulating hormone (FSH) release from pituitary gonadotropes. Additional receptor-mediated actions of the releasing hormone include 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 of cell responsiveness. It is the purpose of this presentation to describe the requirements of gonadotropin release as well as receptor and response regulation in order to prepare an integrated model for these actions of the releasing hormone.

THE INITIAL EVENT IN HORMONE ACTION IS RECOGNITION OF GnRH BY ITS PLASMA MEMBRANE 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). 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, 4, 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. As will be discussed below, however, conditions can be devised in cell cultures which led to diminished cellular responsiveness even in the face of elevated receptor numbers.

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The receptor itself, while still a component of the plasma membrane, has a mol. wt of 136,346 ± 8,120 as measured by target size analysis (Fig. 1 and reference 6).

Occupancy of the plasma membrane GnRH receptor [7] 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, however, structure–activity relationships with Ca^{2+} ion channel antagonists reveal that the channel is not identical to that observed in these other tissues [8].

Patching, capping and internalization

Observations of the cell biology of the receptor can

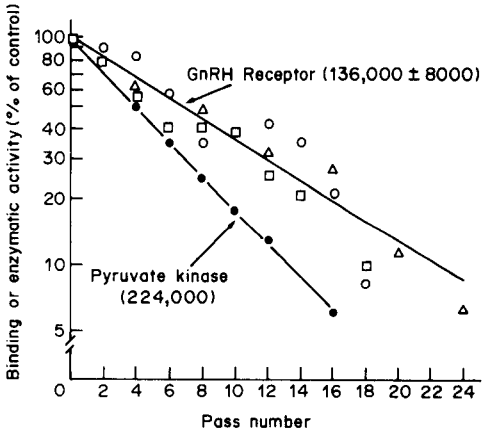

Fig. 1. Target size inactivation of the GnRH receptor. This technique is based on the observation that an inverse relationship exists between the dose dependent inactivation of a macromolecule by ionizing radiation and the size of that macromolecule. "Pass number" indicates the length of time of exposure of purified pituitary cell membranes to ionizing radiation. Compared to standards, one of which is shown (pyruvate kinase), this method provides a molecular weight of 136,346 ± 8,120 for the GnRH receptor. This value is a functional molecular weight for the GnRH receptor while it is still a component of the plasma membrane. In addition the receptor inactivates a single molecular weight class of protein. This is reproduced, with permission of The Endocrine Society, from reference 6.

be made by preparation of fluorescent GnRH analogs which can be monitored on living cells by image intensified microscopy [9, 10]. 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 [11] 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. Studies using similar techniques [12] indicate that GnRH antagonists are also internalized.

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 [13, 14]; 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 affect.

It was apparent that vinblastin could inhibit receptor patching, capping and internalization in response to the releasing hormone but could not inhibit LH release [13]. This also suggested that the process of patching, capping, and internalization could be uncoupled from release. Patching and capping refers 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 [13]. 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 media, 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 appears 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 available. Many of these antagonists appear to work by the classic pharmacologic means; that is, they occupy the receptor but do not produce efficacy (i.e. gonadotropin release). A particular GnRH antagonist was used: D-*p*-Glu^1-D-Phe^2-D-Trp^3-D-Lys^6-GnRH [15]. The substitution of D-amino acids in the first three positions leads to considerable antagonism intrinsic in this molecule. 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 dimer with a very short bridge length (about 12 Å) between the antagonist molecules. 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, i.e. the conversion of a GnRH antagonist to an agonist as a result of its dimerization, was a confusing result. In a number of human disease states antibodies have been identified which crosslink receptors and consequently provide agonist 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 (i.e. univalent "antibody") is coupled with the dimer, we now have a 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 of receptors, could stimulate the response system. 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

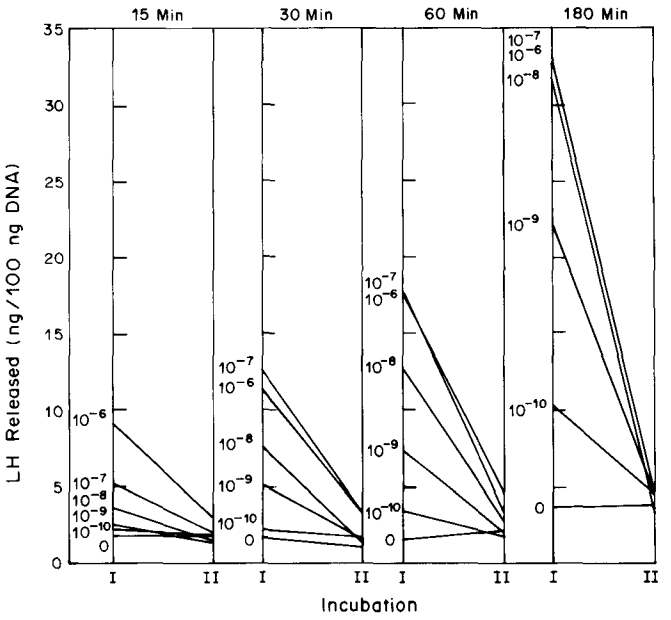

Fig. 2. LH release from rat pituitary cultures in response to GnRH and to GnRH in the presence of D600. Cultures were incubated with the indicated concentrations of GnRH for 15, 30, 60 or 180 min (Incubation I). The medium was then saved for LH RIA and replaced with M199/BSA containing the same concentration of GnRH and 3×10^{-4} M D600. Cells were then incubated for an additional 180 min (Incubation II). This medium was then removed, and LH was determined by RIA. The standard errors, which were less than 10%, are not shown for sake of clarity. Reproduced, with permission of the Endocrine Society, from reference 18.

ability to cross-link receptors, we are now able to see agonist efficacy. 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, 16). 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 super-potentiated 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.

Computer simulations [17] 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 enough, fits the data within approx 5% over 5 dose logs.

RECEPTOR MEDIATED ACTIONS OF THE RELEASING HORMONE

Gonadotropin release: mobilization of extracellular calcium

Gonadotropin releasing hormone (GnRH) stimulates pituitary gonadotropin release by a Ca^{2+} dependent mechanism. Indeed, while it is clear that Ca^{2+} fulfills the requirements of a second messenger, the relative roles of Ca^{2+} mobilized from intra- and extracellular sources have been unclear. A recent study [18] examined the requirements for intra- and extracellular Ca^{2+} by three different means. First, in static cultures a specific Ca^{2+} ion channel blocker, methoxyverapamil (D600), was used to block entry of extracellular Ca^{2+} into pituitary cell cultures in order to determine if brief elevation of intracellular Ca^{2+} (whether derived from external or internal sources) could support continued gonadotropin release (Fig. 2). Studies at a wide range of GnRH concentrations indicated that blockade of Ca^{2+} entry into the gonadotrope (in the presence of continued occupancy of the GnRH receptor by the releasing hormone) resulted in termination of luteinizing hormone (LH) release. Second, compounds which stabilize intracellular Ca^{2+} (preventing its mobilization) such as TMB-8 and dantrolene (Dantrium) were shown not to alter the potency or efficacy of GnRH in stimulating LH release (Figs 3 and 4). Third, the actions of D600 and EGTA were observed in peri-

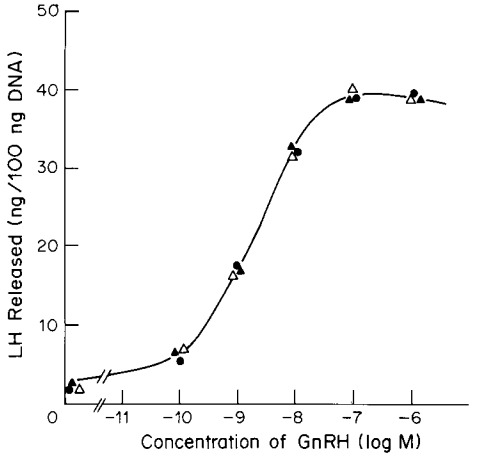

Fig. 3. Effects of TMB-8 on LH release from rat pituitary cultures. Two day cultures were incubated (3 h) and LH assayed as described for Fig. 1. TMB-8 concentrations were 10^{-5} M (open triangle), 10^{-4} M (closed triangle), or 0 (closed circle). Mean values ($n = 3$) are shown. The standard errors, which were less than 10%, are not shown for sake of clarity. Reproduced, with permission of the Endocrine Society, from reference 18.

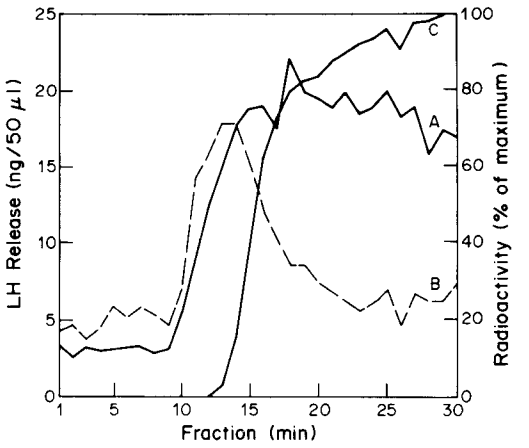

Fig. 5. Effect of D600 on LH release from perifused rat pituitary cultures. Lines A and B show LH release in control (solid line) and experimental (dashed line) columns, respectively. Fractions (200–250 μl) were collected at 1 min intervals. GnRH (10^{-8} M) influx to both sets of columns was added so as to reach the cells at approx fraction 10. Influx of medium 199/BSA containing D600 (3×10^{-4} M) in addition to GnRH into experimental columns was monitored by including approx 2×10^6 cpm/ml $^{45}Ca^{2+}$ in the D600-containing medium (line C). LH and $^{45}Ca^{2+}$ were determined as described in the Experimental Section. Standard errors, which were less than 10%, are not shown for sake of clarity. Reproduced, with permission of the Endocrine Society, from reference 18.

fused cells in order to correlate precisely the release process with access to Ca^{2+} in the extracellular compartment (Figs 5–6). The results of these studies suggest that LH release in response to GnRH is primarily dependent on Ca^{2+} mobilized from extracellular sources. Termination of accessibility to this Ca^{2+} pool also results in termination of release. The data are consistent with a model in which GnRH occupancy of its receptor regulates a plasma membrane Ca^{2+} ion channel; continued access to the

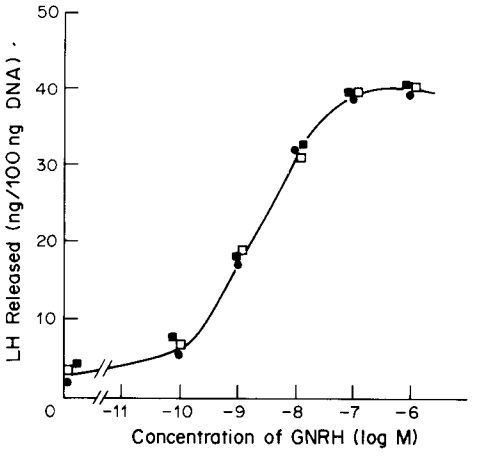

Fig. 4. Effects of dantrolene on LH release from rat pituitary cultures. Two-day cultures were incubated with the indicated concentrations of GnRH and dantrolene for 3 h. At the end of this period, supernatant fluids were removed and assayed for LH by RIA. Dantrolene concentrations were 2.5×10^{-4} M (open square), 1.3×10^{-3} M (solid square), or 0 (solid circle). Mean values ($n = 3$) are shown. The standard errors, which were less than 10%, are not shown for sake of clarity. Reproduced, with permission of the Endocrine Society, from reference 18.

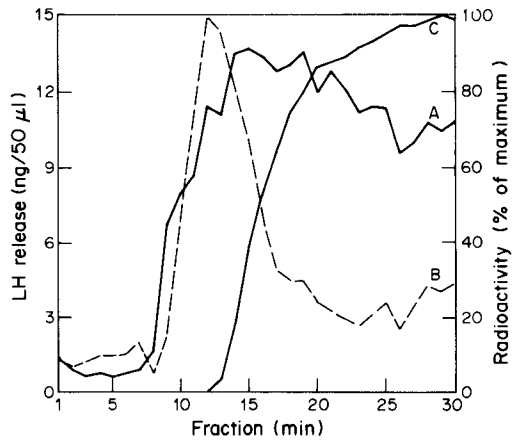

Fig. 6. Effect of EGTA on LH release from perifused rat pituitary cultures. Lines A and B show LH release in control (solid line) and experimental (dashed line) columns, repsectively. Fractions (200–250 μl) were collected at 1 min intervals. GnRH (10^{-8} M) influx to both sets of columns was started so as to arrive at the perifusion chamber at fraction 10 (as nearly as possible). Influx of M199/BSA containing EGTA (4 mM) in addition to GnRH into experimental columns was monitored by including approx 2×10^6 cpm/ml $^{45}Ca^{2+}$ in the EGTA-containing medium (line C). LH and $^{45}Ca^{2+}$ were determined as described in Experimental Section. Standard errors, which were less than 10%, are not shown for sake of clarity. Reproduced, with permission of the Endocrine Society, from reference 18.

extracellular Ca^{2+} pool is required for continued LH release.

The requirement of Ca^{2+} have been clearly established [19, 20]; 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. GnRH provokes calmodulin disappearance in the cytosolic fraction and appearance in the plasma membrane fraction [21]. The constitutive expression of calmodulin in these cells suggests that the redistribution may actually reflect translocation within the cells.

A related observation is that calmodulin inhibitors [22], some of which are highly specific [23], 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 [21].

Regulation of cell responsiveness

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, 24). 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 [24]. This provides a good model system for kinetic studies. 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 (i.e. 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 [25]. 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 of this reduced sensitivity. This also suggested that secretion and desensitization may be mediated by fundamentally different processes in this system.

In order to prove this question further, advantage was taken of the fact that extracellular calcium is an absolute requisite for GnRH stimulated LH response from these cells [26]. 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. 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 chemically fundamentally different means. Further, it 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. Occupancy had to be by an agonist in order for desensitization to occur. Therefore, in comparing desensitization with the release process we find that the release process has an absolute requirement for calcium while desensitization does not. We find that both systems require occupancy by an agonist; an antagonist is not satisfactory. It was, at this point, desirable to see if the dimerized antagonist could provide desensitization. Indeed, it was able to do so [27]; 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 GnRH receptor

Pituitary cell cultures were used to examine the effect of GnRH and other treatments on the GnRH receptor [28]. 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. Upregulation, 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, [28]) 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 [28, 29]. 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 [30] implicating such an action at low concentrations (ED_{50} about 100 μM).

Table 1. 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
Intracellular Ca^{2+}	Yes	No	Yes
Requires:			
Time	0–3 h	0–3 h	5–10 h
Protein synthesis	No	No	Yes
Intracellular 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.

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 [31]. 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 crosslink GnRH receptors and mimic GnRH actions. The requirement of gonadotropin release, receptor regulation and regulation of cell responsiveness is shown above in Table 1.

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 [32–33] and others arguing against one [34–35]. The present study suggest that during the period of receptor recovery (5–10 h), when the cells are clearly refractory to GnRH [36], 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 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 [37]) but not an M site. It becomes an agonist when it is (artifically) 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. A related type of compound might have a specificity component conferred upon it by recognition of a site other than the active site (i.e. that which recognizes the GnRH molecule). 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. Polycations (particularly polylysine of large size classes) may also be an example of a secretogogue which contains an "M" but not "R" site [38].

Considerable evidence exists which suggests that the R and M sites correspond to definite physical domains of the GnRH molecule: p-Glu^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 (i.e. des-His^2-GnRH) resulted in a molecule which bound to the GnRH receptor (albeit with lower affinity) but which had no LH releasing activity. This molecule first demonstrated the potential of synthesizing GnRH analogs which behaved as antagonists.

Further studies [39] 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 p-Glu^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 10 amino acids have not been identified which bind with appreciable affinity. This observation may emphasize the importance of the first and tenth amino acids in receptor recognition.

CONCLUSION

The available data support a model shown in Fig. 6 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 one hand, appear to be Ca^{2+} independent while gonadotropin release and GnRH receptor up-regulation are Ca^{2+}-mediated actions.

Note added in proof: Since submission of this article it has been possible to show that activators of protein kinase C [40] such as phorbol esters [27] and diacylglycerols [40] act synergistically [41] with Ca^{2+} ionophore A23187. This presents the possibility that endogenous PKC activators may have a role in modulation of GnRH action.

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