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Gonadotropin-releasing hormone stimulation of luteinizing hormone release: A ligand–receptor–effector model

(microaggregation/ligand internalization/patching/capping)

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ABSTRACT **A divalent antibody conjugate of a pure antagonist of gonadotropin-releasing hormone (GnRH) behaved as an agonist—i.e., released luteinizing hormone (LH) from pituitary cultures. Release was measured over a wide range of conjugate concentrations; it rose to a maximum of 66% of the LH released by the optimal concentration of GnRH and declined to basal levels at very high concentrations. This behavior was modeled on the assumption that the antibody conjugate, A, can react with a receptor, R, to form a complex, A·R, which in turn can react with another receptor to form $A{\cdot}R_2$. This dimer then can react with a quiescent effector, E (e.g., a closed Ca^{2+} ion channel), to form $A{\cdot}R_2{\cdot}E$, which contains activated effector and leads to cellular responses. The equilibrium equations governing the behavior of this model were derived, solved, and found to yield a good fit to the experimental data. Consideration of our data in this model system, and of other available data describing the behavior of ligands in other cells, suggests that the present model may be of wide applicability.**

Gonadotropin-releasing hormone (GnRH; <Glu[1]-His[2]-Trp[3]-Ser[4]-Tyr[5]-Glu[6]-Leu[7]-Arg[8]-Pro[9]-Gly[10]-NH_2) stimulates luteinizing hormone (LH) release from the anterior pituitary by a receptor-mediated, calcium-dependent mechanism involving calmodulin (1–3). [D-<Glu[1]-D-Phe[2]-D-Trp[3]-D-Lys[6]]GnRH (GnRH-Ant) is a competitive antagonist that inhibits GnRH-stimulated LH release (4). This antagonist can be dimerized with ethylene glycol bis succinimidyl succinate (EGS). Like the monomer, the dimer was a pure antagonist. When crossreacting antibody (AB) was incubated with excess dimer, a divalent antibody with a GnRH-Ant dimer attached to each arm ("conjugate") was formed. One molecule of each GnRH-Ant dimer was tightly bound to each antigenic binding site and unavailable to the receptor. The two molecules of GnRH-Ant available to the receptor are separated by about 150 Å. In contrast to the parent compounds, the divalent antibody–dimer conjugate was a potent *agonist* (4). The reduced pepsin fragment of the conjugate (monovalent) was not an agonist. The conversion of an antagonist to an agonist by coupling to a divalent antibody suggested that the formation of a receptor microaggregate was sufficient to stimulate signal transduction across the plasma membrane. Receptor microaggregation (or perhaps dimerization alone) is involved in a large number of hormone, immune, and drug receptor systems (for review, see ref. 5 and below).

Preliminary studies of the potency of the divalent conjugate (AB-[$(GnRH\text{-}Ant)_2$-EGS]$_2$) indicated that when conjugate concentration was raised above an optimally effective level, LH release declined. Minton (6) has proposed a quantitative model for hormone action based on a divalent ligand hypothesis. In this model, a single hormone molecule has two distinct regions: a specificity determinant that binds with high affinity to cell surface receptors and a response determinant that binds with lower affinity and specificity to a second class of sites called effectors. Although this divalent ligand model has many attractive features, it seemed unlikely to be applicable to the analysis of the release of LH by the AB-[$(GnRH\text{-}Ant)_2$-EGS]$_2$ conjugate, because GnRH-Ant is a decapeptide whose largest dimension is shorter than the ≈150-Å separation of the accessible GnRH-Ant molecules in the conjugate.

In the present work we describe a mathematic model of hormone action that quantitatively accounts for the release of LH by the antibody conjugate over a wide range of concentrations.

MATERIALS AND METHOD

AB-[$(GnRH\text{-}Ant)_2$-EGS]$_2$ conjugate was prepared as described (4) except that the reaction time was increased to 7 hr. Rat pituitary cell cultures (7) were exposed to serial dilutions of the purified conjugate for 5 hr (maximal stimulation) and LH release was determined by radioimmunoassay (7).

RESULTS

Fig. 1*A* shows the amount of LH released over a wide range of concentrations of the divalent antibody conjugate AB-[$(GnRH\text{-}Ant)_2$-EGS]$_2$. It can be seen that the response (i.e., release of LH) rises to a maximum of about 66% of the response given by a maximally stimulating concentration of GnRH (10^{-7} M) and then, with increasing concentrations of conjugate, falls to near baseline levels.

The Hormone–Receptor Dimer–Effector Model. A simple model that explains the experimental data shown in Fig. 1 postulates that when the divalent conjugate interacts with two receptors, it positions them at an appropriate distance for interaction with an effector. This ternary complex leads to the biological response. For simplicity, we assumed (consistent with experimental evidence; ref. 8) that there was no appreciable change in cell responsiveness during the period of the bioassay (5 hr).

Let [A] = the concentration of AB-[$(GnRH\text{-}Ant)_2$-EGS]$_2$ and A_T is the total amount of antibody conjugate added; [R] = the concentration of free receptor molecules and R_T is the total amount of receptors present; [A·R] = the concentration of antibody–conjugate molecules that have only a single receptor bound; [$A{\cdot}R_2$] = the concentration of antibody–conjugate molecules with two receptors bound, one to each of the two available GnRH-Ant molecules; [E] = the concentration of free ef-

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Abbreviations: GnRH, gonadotropin-releasing hormone; GnRH-Ant, [D-<Glu[1]-D-Phe[2]-D-Trp[3]-D-Lys[6]]GnRH, a competitive antagonist that inhibits GnRH-stimulated luteinizing hormone release; LH, luteinizing hormone; EGS, ethylene glycol bis succinimidyl succinate; LATS, long-acting thyroid-stimulating; AB, antibody.

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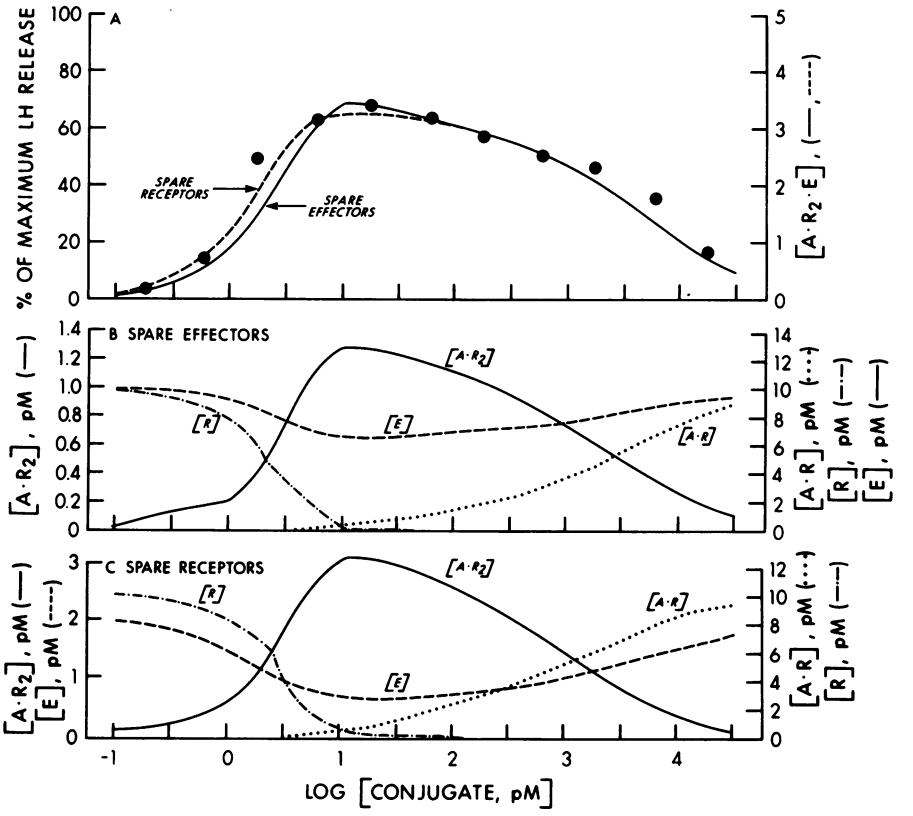

FIG. 1. LH release as a function of the concentration of the divalent antibody conjugate AB-[(GnRH-Ant)$_2$-EGS]$_2$. Pituitary cultures were incubated for 5 hr in 1 ml of medium with the indicated concentrations of antibody conjugate and the amount of LH released was assayed as described. The solid symbols represent the average value for paired cultures. Antibody–conjugate concentration was arbitrarily defined so that 10 pM corresponded to the concentration yielding maximal release of LH; in this experiment, 100% release of LH was defined as the amount of LH release by 10^{-7} M GnRH. (*A*) Two theoretical fits to these data are shown. Parameters for the fit labeled "spare receptors" (----) were: R_T = 10 pM; E_T = 2 pM; K_1 = 0.18 pM^{-1}; K_2 = 10 pM^{-1}; K_3 = 0.6 pM^{-1}. Parameters for the fit labeled "spare effectors" (——) were: R_T = 10 pM; E_T = 10 pM; K_1 = 0.2 pM^{-1}; K_2 = 10 pM^{-1}; K_3 = 0.4 pM^{-1}. (*B* and *C*) The computed values of [E], [R], [A·R], and [A·R$_2$] for the spare effectors and spare receptors fits shown in *A*. For further details see text.

fector molecules (e.g., Ca^{2+} ion channels) and E_T is the total amount of effectors present; [A·R$_2$·E] = the concentration of active effector molecules. The amount of response (e.g., LH release) is assumed to be directly proportional to [A·R$_2$·E]. Fig. 2 presents a highly schematic representation of these species.

If the system is assumed to be at equilibrium, then the following equations are applicable:

$$A + R \rightleftharpoons A{\cdot}R \qquad K_1 = [A{\cdot}R]/[A][R]; \qquad [1]$$

$$A{\cdot}R + R \rightleftharpoons A{\cdot}R_2 \qquad K_2 = [A{\cdot}R_2]/[A{\cdot}R][R]; \qquad [2]$$

$$A{\cdot}R_2 + E \rightleftharpoons A{\cdot}R_2{\cdot}E \qquad K_3 = [A{\cdot}R_2{\cdot}E]/[A{\cdot}R_2][E]. \qquad [3]$$

Conservation of mass requires that:

$$A_T = [A] + [A{\cdot}R] + [A{\cdot}R_2] + [A{\cdot}R_2{\cdot}E]; \qquad [4]$$

$$R_T = [R] + [A{\cdot}R] + 2[A{\cdot}R_2{\cdot}E] + 2[A{\cdot}R_2]; \qquad [5]$$

$$E_T = [E] + [A{\cdot}R_2{\cdot}E]. \qquad [6]$$

As discussed by Minton (6) and Reynolds (9), the association constant K_1 may be written as $2K_1'$, in which K_1' is the intrinsic affinity constant of one of the available molecules of GnRH-Ant on the divalent antibody conjugate for the receptor, and K_2 may be written as $\alpha K_1'/2$, in which α is a localization factor that reflects the increase in effective concentration of the second ligand of the conjugate due to the binding of the first ligand of the conjugate to the surface. Eqs. 1 through 6 can be solved algebraically to yield a sixth-order polynomial in [R]. The real roots of this polynomial were found by an iterative method. The concentrations of all species then could be determined.

The qualitative response characteristic of this model can be simply described. At low levels of bivalent agonist, the response will increase rapidly as pairs of receptors are positioned the correct distance apart to interact with effector. With increasing concentration of the conjugate, there will be an increasing probability of formation of antibody complexes with only one receptor bound (see also ref. 9), and, because suitably positioned pairs of receptors are required to interact with (and activate) the effector, the response will decline, as observed experimentally.

As a preliminary step to fit the model to the data presented in Fig. 1*A* it was necessary to estimate some of the parameters. Binding studies have shown that there are about 10^4 receptors for GnRH per gonadotrope cell (10, 11) and in the experiment of Fig. 1*A* there were $\approx 5 \times 10^5$ cells per ml, yielding a value of $\approx$8 pM for R_T. We further assume that the concentration of antibody–antagonist conjugate in the experiment shown in Fig. 1*A* ranged from about 0.3 to 30,000 pM. Because the exact concentration could not be determined, we could have either varied R_T to fit an arbitrarily assumed concentration of the antibody or chosen a fixed value for the receptor concentration and matched the theoretical curves by adjusting the concentration of the original antibody so that the experimental curve moved slightly to the left or right along the abscissa in Fig. 1. We chose to set R_T = 10 pM and adjusted the antibody concentrations by 0.25 log units from the concentrations that had been initially assumed. The association constants K_1, K_2, and K_3 were likely

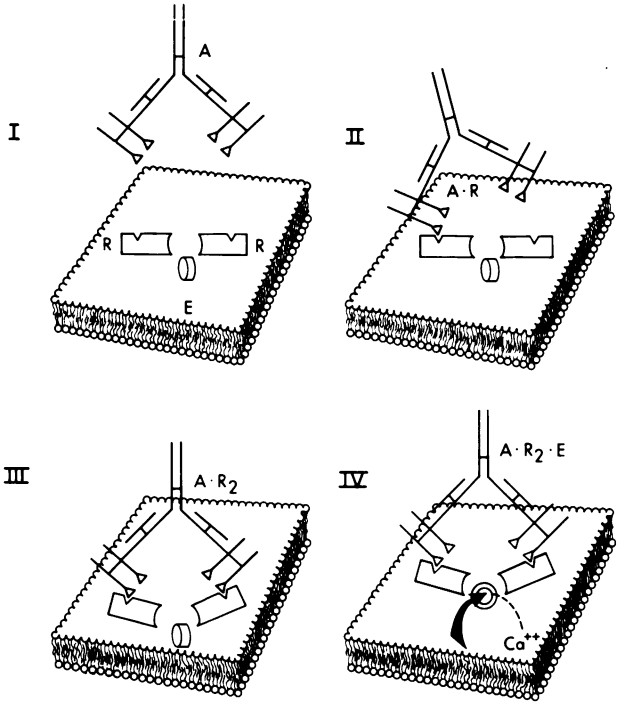

FIG. 2. Schematic diagram of possible molecular species in a system containing a divalent antibody conjugate, A (AB-[(GnRH-Ant)$_2$-EGS]$_2$), a receptor, R, and an effector, E, which is here represented as a channel for the passage of Ca^{2+} ions but in other cell systems could be a nucleotide cyclase or other generator of a second messenger.

to range between 0.1 and 50 pM^{-1}.

It turned out that the data could be fit by two very different choices of effector concentration, E_T = 2 pM (Fig. 1A, dashed line) and E_T = 10 pM (Fig. 1A, solid line). Given that R_T = 10 pM, the maximal possible concentration of $A{\cdot}R_2{\cdot}E$ (the active effector; e.g., an open Ca^{2+} channel) with E_T = 2 pM is 2 pM, leaving 6 pM free receptors out of an original 10 pM, corresponding to 60% spare receptors. This is close to experimentally determined numbers of spare GnRH receptors in pituitary cells (figure 11 in ref. 10). With R_T = 10 pM and E_T = 10 pM, the maximal possible $[A{\cdot}R_2{\cdot}E]$ is 5 pM, corresponding to 50% spare effectors. Although this case does not apply to the interaction of GnRH (and of AB-[(GnRH-Ant)$_2$-EGS]$_2$) with pituitary cells from immature rats, it may describe some other ligand–target cell interactions. We shall discuss these two cases separately.

Case 1: Spare Receptors. The parameter values used for the fit (Fig. 1A, dashed line) to the data were: R_T = 10 pM; E_T = 2 pM; K_1 = 0.18 pM^{-1}; K_2 = 10 pM^{-1}; K_3 = 0.6 pM^{-1}. The ratio between K_2 and K_1 is equivalent to a localization factor, α, of 220. Fig. 1C shows the concentrations of [E], [R], [A·R], and $[A{\cdot}R_2]$ as a function of antibody–antagonist conjugate. At the lowest concentration of antibody, 9.8 pM out of 10 pM receptors are free. As the concentration of conjugate increases, the concentration of free receptors begins to decrease and the concentrations of $[A{\cdot}R_2]$ ("dimerized" receptor) and of $[A{\cdot}R_2{\cdot}E]$ (see Fig. 1A) increase in parallel while that of [A·R] remains extremely low. Thus, the parameters required for a good fit to the data imply that, at low conjugate concentration, dimeric complexes, $A{\cdot}R_2$, are favored over the monomeric receptor–antibody species, A·R, and, furthermore, that the concentration of active effector, $[A{\cdot}R_2{\cdot}E]$, is nearly proportional to that of $[A{\cdot}R_2]$. As the concentration of added conjugate increases past that required for maximal agonist activity, the concentration of A·R becomes appreciable and rises steadily until at very high conjugate concentrations almost all of the receptors are in the monomeric (A·R) form and agonist activity has dropped to almost basal levels.

Fig. 3 shows the effects of small changes in each parameter individually on the shapes of the computed curves. Increasing K_3, the affinity of the receptor–antibody dimer to the effector, increases the response at all concentrations of antibody conjugate, whereas decreasing K_3 leads to a marked reduction in maximal response. It should be noted that the present model provides a simple explanation for the ability of certain hormone analogues to behave as partial agonists and for the decrease in agonist activity as the analogue concentration is increased above that required for optimal response. Experiments with partial agonists also are frequently performed over a relatively limited range of agonist concentrations. Much additional insight could be gained if such experiments were carried out to very high concentrations of the partial agonist.

When there is a considerable excess of receptors relative to effectors, the total concentration of effector, E_T, has only limited influence on the shape of the response curve (Fig. 3*B*). Thus, effector concentration could vary from 1 to 3 pM in this system with little effect on the shape of the curve of LH release. This feature may explain the prevalence of spare receptors in most hormone responsive systems that have been examined, because the cellular response to a change in hormone concentration would remain about the same even if effector concentration was not closely controlled.

The primary effects of variation in K_1 and K_2 are on the shape of the response curves at high conjugate concentration. Increasing K_1 (i.e., increasing formation of monomeric species A·R) favors a more rapid loss of agonist activity at high conjugate

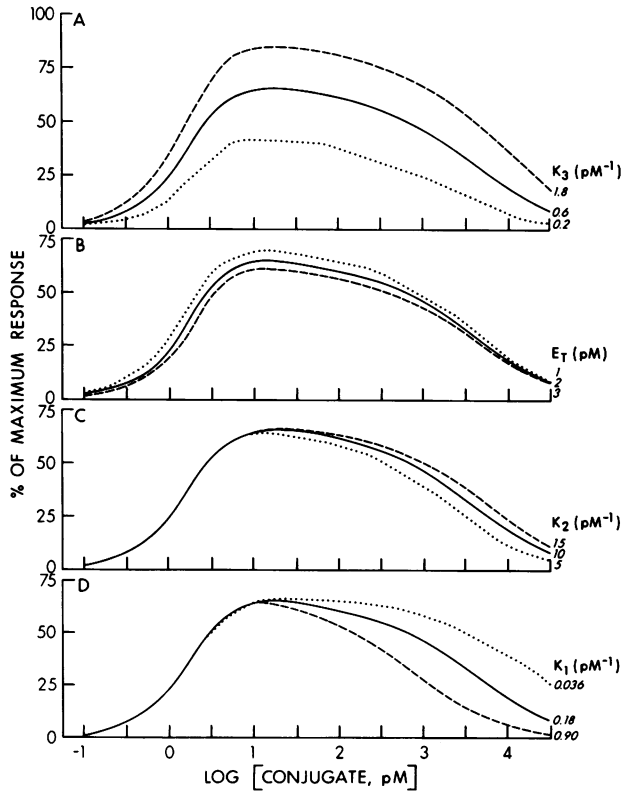

FIG. 3. Effect of varying individual parameters on the response characteristics of pituitary cells with spare receptors to the addition of antibody conjugate. Except as otherwise indicated, parameters are: R_T = 10 pM; E_T = 2 pM; K_1 = 0.18 pM^{-1}; K_2 = 10 pM^{-1}; K_3 = 0.6 pM^{-1}. For further details, see text.

concentrations (Fig. 3C). A similar result is obtained when K_2 is decreased (Fig. 3D). In the absence of further independent information on the magnitude of K_1, K_2, or K_3, there is some latitude in the choice of parameter values used to fit the data in Fig. 1A and we emphasize that the values used are not tightly constrained. Nevertheless, the model does provide useful approximate values for K_1, K_2, and K_3, and it may be expected that further studies will allow more precise estimates to be obtained.

Case 2: Spare Effectors. The parameters used for the fit shown by the solid line in Fig. 1A were R_T = 10 pM; E_T = 10 pM; K_1 = 0.2 pM^{-1}; K_2 = 10 pM^{-1}; K_3 = 0.4 pM^{-1}. Except for E_T, these parameters are close to or identical with those used to obtain the fit to the data when E_T was 2 pM—i.e., when there was an excess of receptors over effectors. Comparison of Fig. 1B to Fig. 1C shows that the shapes of the curves for [R], [A·R_2], and [A·R_2·E] closely resemble one another, consistent with the fact that both sets of parameters yield a good fit to the experimental data. The effects of varying K_1, K_2, K_3, and E_T (the latter in the range $E_T > 0.5\ R_T$) are virtually identical to those shown in Fig. 3, for the case in which $E_T < 0.5\ R_T$ (Fig. 4). Thus, the two seemingly quite different systems are remarkably similar both in their response characteristics (e.g., LH release) and in the detailed behavior of the receptor and effector complexes. An independent measure of receptor occupancy relative to physiological response is required (as in the present case) to determine whether a spare receptor or spare effector fit is appropriate.

DISCUSSION

We recently proposed a "three-step mechanism" as a model for GnRH action (12): (*i*) GnRH initially binds to its plasma membrane receptor; (*ii*) Ca^{2+} is mobilized, through a Ca^{2+}-conducting channel; and (*iii*) gonadotropin is released from preexisting pools. The observations that GnRH caused a redistribution of calmodulin in the gonadotrope (2) and that GnRH-stimulated gonadotropin release was inhibited by anti-calmodulin drugs (3) have suggested that calmodulin is the intracellular Ca^{2+} receptor linking steps *ii* and *iii*.

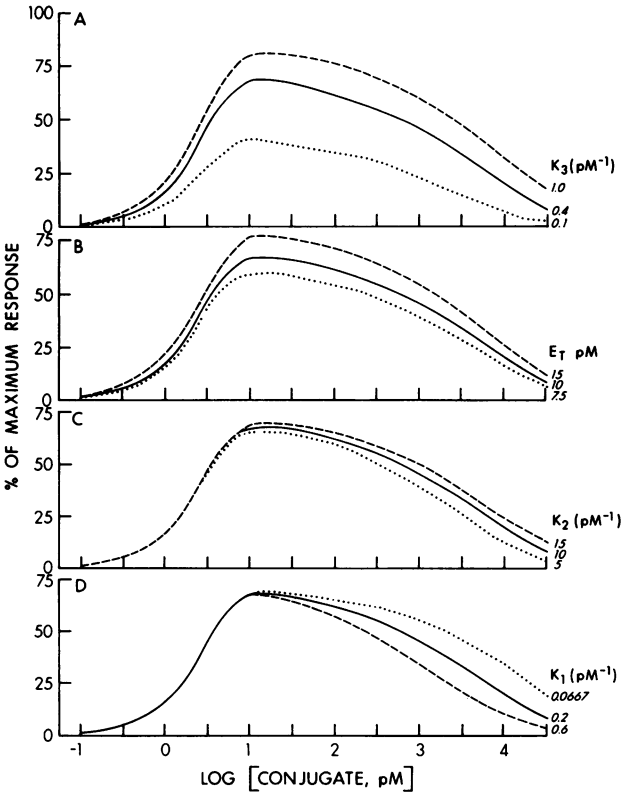

FIG. 4. Effect of varying individual parameters on the response characteristics of pituitary cells with spare effectors to the addition of antibody conjugate. Except as otherwise indicated, parameters are: R_T = 10 pM; E_T = 10 pM; K_1 = 0.2 pM^{-1}; K_2 = 10 pM^{-1}; K_3 = 0.4 pM^{-1}.

Some insight into the links between steps *i* and *ii* came from the finding (4) that the potency of an *agonist*, (D-Lys^6-GnRH)$_2$-EGS, was markedly potentiated upon addition of a crossreactive antibody. These results strongly suggested that formation of a receptor microaggregate (perhaps dimer) was required to evoke responses. It also was noted (figure 3 of ref. 13) that with increasing concentrations of antibody, the enhancement of LH release reached a maximum and then declined towards basal levels. Further information linking steps *i* and *ii* comes from the observations (4) that a GnRH antagonist was converted to an agonist when the ability to crosslink receptors was conferred on it by binding onto a divalent antibody. The present formalism, developed to explain the behavior of the antibody–antagonist conjugate, also provides a straightforward explanation for the behavior observed when the potency of the agonist (D-Lys^6-GnRH)$_2$-EGS was increased by the addition of a crossreacting antibody.

Thus, all observations involving the use of antibodies—whether coupled to analogues that by themselves have agonist or antagonist activity—are consistent with this model in which receptor occupancy is coupled to effector activation. The model is based on the premise that the antibody conjugate bridges two receptors, thereby enhancing the probability of receptor dimerization in the lateral plane of the plasma membrane. This complex (A·R_2) interacts with the quiescent effector E, thereby activating it (A·R_2·E). The present model differs from that of Minton (6) in that a hormone (or other agonist or partial agonist) is considered to be a *monovalent* molecule with high affinity and high specificity for the receptor. The unoccupied receptor and the receptor–hormone monomer have only a low affinity for the effector; in the present model, this interaction is assumed to be negligible. To keep this model as simple as possible, we also have ignored any possible conformational changes in the receptor (for which there is no evidence in the present system) as a result of binding of an agonist molecule, and we have implicitly assumed that the consequences of receptor aggregation to form trimers or higher n-mers can be neglected and that receptors (and effectors) are not removed from the membrane by capping or patching reactions during the time of the experiment.

Large scale patching, which can be viewed by image-intensified microscopy (14), can be inhibited by vinblastin, a drug which does not inhibit GnRH-stimulated LH release (15). This and other observations (16) indicated that large scale aggregation is not required for release, whereas microaggregation appears to be driven by receptor–receptor interactions. Microaggregation may be the driving force for activation of the effector, whereas large scale aggregation and internalization may promote extinction of responses.

Because the present simple model yields a satisfactory fit for LH release by the antibody–antagonist conjugate, the question arises as to how GnRH would cause LH release. One possibility is that GnRH reacts with receptor and a pair of these interacts with effector. However, other pathways to form this active complex also are possible. A description of kinetic models in use to explain hormone-elicited response has appeared recently (17).

It is clear that other systems share aspects in common with that described in the present study. We first consider the case of autoantibodies to the insulin receptor. The monovalent Fab fragments and the divalent antibodies are both competitive antagonists of insulin binding, but only the latter mimic the action

of insulin in glucose transport (18). Furthermore, the inactive Fab fragments regain insulin-mimicking activity in the presence of a crosslinking antibody raised against the monovalent Fab fragments (19). Such behavior is obviously similar to that described in the present study.

Jacobs *et al.* (20) prepared an antibody to insulin receptors that did not compete with ^{125}I-labeled insulin for binding to the receptor but did precipitate the solubilized receptors and had insulin-like activities. Thus, the actions of both autoantibodies to insulin receptors and antibodies prepared against purified insulin receptors are in accord with the model described in the present paper. Maturo and Hollenberg (21) isolated two classes of insulin receptors. One appears to be identical to a highly purified insulin receptor, whereas the other has a high affinity for insulin and appears to be complexed to a glycoprotein. As noted by Minton (6), the receptor–glycoprotein complex may be the receptor–effector complex. However, it is not necessary that there be only one type of effector for a given kind of receptor. Whereas low concentrations of insulin activate glucose transport, higher concentrations activate amino acid transport (22). Such behavior could indicate different classes of insulin receptors but would follow directly from the present model by having a single population of receptors that interact with high affinity to a glucose transport effector and a lower affinity to an amino acid transport effector.

A variant on this theme occurs when immunoglobulins interact with receptors on platelets. IgG monomers have low binding affinity (23). When two IgG molecules are crosslinked, binding to monocytes, polymorphonuclear leukocytes (24, 25), and platelets (23) increases markedly. That tetramers are somewhat more active than dimers suggests that the optimal spacing is greater than has been achieved by the crosslinking agent used to form the dimers, although other explanations are possible. The necessity for dimer formation as a prerequisite to interaction with an effector also has been deduced by Foreman (26). In this system, binding of IgE to its receptor in the mast cell membrane does not bring about histamine secretion, but an antibody against the Fc region of IgE crosslinks adjacent IgE molecules and induces histamine secretion, as does crosslinking by other procedures (26). The essential role of receptor dimers as the fundamental unit of signal transmission for histamine release also has been recognized by DeLisi and Siraganian (27), who have developed a theoretical treatment (see also refs. 28 and 29) that can account for the kinetics (and hence, the equilibrium states) of cell surface receptors that interact with divalent ligands. However, they do not specifically incorporate the interactions of the receptor–agonist dimer with an effector molecule.

Much work has been done recently on the immunoglobulin responsible for the long-acting thyroid-stimulating (LATS) properties of sera of certain patients. Endo *et al.* (30) have purified the IgG molecules from plasma and found that they retain LATS activity as well as the capacity to stimulate adenyl cyclase and to inhibit the binding of ^{125}I-labeled bovine thyroid-stimulating hormone. Of particular interest in the present context is the statement by Mehdi and Kriss (31) that "native unlabeled TSI (LATS) stimulated adenyl cyclase activity at concentrations in the range of 5–50 μg/tube, produced no further increase between 50–500 μg/tube, and produced submaximal stimulation at concentrations above 500 μg/tube (points not shown)." It seems possible that long-acting thyroid stimulation may cross-link receptors that in turn interact with an effector as in the model postulated here.

Insofar as the present model accounts for the action of AB-$[(GnRH\text{-}Ant)_2\text{-}EGS]_2$ and appears consistent with the behavior of antibodies in systems as diverse as gonadotropes, adipocytes, hepatocytes, thyroid cells, platelets, and mast cells, among others, it appears that this model may be of wide applicability.

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