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Hormone Receptor Interactions at the Cell Membrane

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I. Introduction

Many hormones, neurotransmitters, drugs, and cellular toxins initiate their action via specific interactions with plasma membrane receptors. It is particularly striking how important the in-depth studies of a variety of cellular toxins have been to the development of the "receptor" concept in general and to the detailed examination of receptors for certain endogenous substances. For example, at about the turn of the century, studies of the actions of curare and nicotine led Langley (65) to postulate the existence of a "receptive substance" in cells. At about the same time, work by Ehrlich (32) on tetanus toxin and on the antimicrobial action of a series of triphenylmethane dyes led him to formulate the receptor concept in terms of families of compounds that act at the same cellular receptor site. Further work by Clark (16, 17), using atropine and acetylcholine, led to the realization that the receptors for these active substances were present in very small numbers and that the receptors were most likely localized at the cell membrane. Subsequent to this early work, studies with muscarine, atropine, and nicotine delineated the two major classes of receptors for acetylcholine, the so-called "muscarinic" and "nicotinic" receptors; more recently, the use of the snake venom toxin, α-bungarotoxin, has been instrumental in studying the detailed molecular characteristics of the nicotinic cholinergic receptor. Furthermore, relatively recent studies of the binding and action of atropine at the muscarinic acetylcholine receptor in smooth muscle by Paton and Rang (80a) not only confirmed rather strikingly the early quantitative estimates of Clark (16, 17), but set the stage for an ever enlarging number of studies of the binding of ligands to putative membrane receptors. Thus, in large part, our knowledge concerning the receptors for acetylcholine has come from a detailed study of the action of a variety of toxic substances. Likewise, it is the case that toxins are proving of enormous value in the study of the receptor mechanisms related to the action of other agents, for example, histrionicotoxin in the study of neurotransmitter-regulated sodium channels and cholera toxin in relation to hormone-stimulated adenylate cyclase.

At the present time, the study of hormone receptor mechanisms is entering an exciting phase. Over the past decade or so experimental expertise has been developed sufficiently to measure with confidence the binding of radioactive ligand probes to high-affinity, low-capacity binding sites both in intact cells and in particulate and soluble membrane preparations. Reference can now be made to several comprehensive reviews and treatises dealing both with the details of the methodologies involved and with a large amount of information about many neurotransmitters and hormones (9, 10, 23, 34, 59, 66, 67, 92, 117). The challenge that presents itself now is to unravel the undoubtedly complex processes that translate the initial recognition of a ligand by a cell into a final cellular response. Whereas ligand binding studies have provided a remarkably good "first look" at the problem of receptor function, it is likely that new

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approaches will be necessary to elucidate further the membrane-localized mechanisms involved in hormone action. It is the object of this review to highlight some of the new directions concerned with hormone action that have developed.

II. Membrane Fluidity and the Mobile Receptor Paradigm of Hormone Action

A. Development of the Model

As reviewed elsewhere (72, 104, 105) and in this symposium, there has been considerable progress in understanding the organization of the cell membrane. It has been realized for some time now, based on fluorescence and electron microscopic observations, that certain membrane proteins are free to diffuse in the plane of the plasma membrane. It was a logical extension of such observations to propose that receptors for hormones would also be mobile constituents that could interact with other elements in the plane of the membrane. The "mobile" or "floating" receptor model, developed separately by Cuatrecasas and colleagues (5, 21, 23, 55, 56), by DeHaen (24), and by Boeynaems and Dumont (11) proposes that a hormone-receptor complex may interact with a number of "effector" substituents in the plane of the membrane, as depicted in Figure 1. In large part, the genesis for the theory centers around the observation that a number of receptor-specific agonists (catecholamines, prostaglandins, glucagon, ACTH, etc.) can independently stimulate adenylate cyclase in a cell such as the adipocyte in a manner indicative of a unique adenylate cyclase enzyme

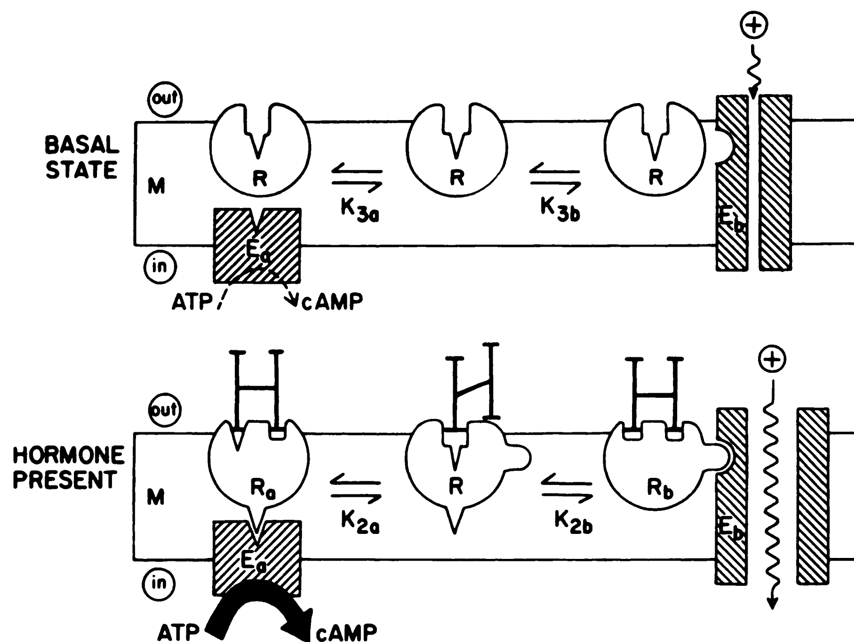

FIG. 1. The mobile receptor model of hormone action. The ability of the hormone-receptor complex to interact in the plane of the membrane is shown for two effector macromolecules: E_a = adenylate cyclase, converting adenosine 5′-triphosphate to adenosine 3′,5′ cyclic monophosphate (cAMP); E_b = a transport system for a monovalent cation (depicted as a closed or open membrane pore). The equilibria are portrayed schematically in terms of the equilibrium constants expressed by equations (2) and (3), both in the absence (*upper*) and presence (*lower*) of a hormone agonist, H. The differences in hormone affinities between the uncomplexed and complexed states (receptor conformations R_a and R_b) are indicated by different "lock and key" configurations; the possible (weak) association between receptor and effectors in the absence of hormone is also shown. Only the ternary complex (HRE) is fully active, either converting ATP to cyclic AMP at an accelerated rate (HR_aE_a) or opening an ion channel (HR_bE_b). A receptor site for the binding of an inhibitor to form a nonassociating complex, IR (not shown), is also depicted. "out" = outside of cell; "in" = inside of cell; M = cell membrane.

responding in a complex way to various hormonal stimuli. Rather than supposing that all of the receptors are clustered about the cyclase enzyme complex or that each receptor is physically associated with its own cyclase, it is proposed that each independent hormone-receptor moiety can freely compete for the effector (adenylate cyclase) in the plane of the membrane. The model, as depicted in Figure 1, does not restrict the number of effectors with which the hormone-receptor complex may interact (e.g., an ion transport channel may be perturbed as well as a membrane enzyme complex) and the model is generally applicable to the modulation of any membrane process by a variety of hormones. Additionally, the model does not preclude the self-association of one hormone-receptor complex with another to form a receptor cluster.

B. Predictions Based on the Mathematical Analysis of the Model

In a simplified version, the equilibria involved in the mobile receptor model can be expressed:

$$H + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} HR \qquad K_1 \quad (1)$$

$$HR + E \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} HRE \qquad K_2 \quad (2)$$

where H, R, and E represent hormone, receptor, and effector (e.g., membrane-bound adenyl cyclase) respectively. It is evident that the equilibria could readily be made more complex, so as to account for cooperative phenomena, by varying the stoichiometry of the reacting species. For example, either the receptor or effector may well represent oligomeric macromolecular species. Indeed, should receptors exist as clusters within the plane of the membrane, negative cooperativity between receptor molecules alone could account for "spare" receptors as discussed by Levitzki (69), whereby low ligand occupancy would lead to a large configural change of the receptor cluster. In equations 1 and 2, the values K_1 and K_2 represent the microscopic equilibrium association constants for the reactions with overall forward and reverse rate constants, k_1, k_{-1}, k_2, and k_{-2}. It is a fundamental hypothesis of the mobile receptor model that the affinity of the hormone-receptor complex for the effector, as expressed by the equilibrium constant, K_2, is greater than the affinity of the uncomplexed receptor for the effector, as given by the following equation:

$$R + E \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} RE \qquad K_3 \quad (3)$$

and expressed by the equilibrium constant K_3. It is thus proposed that for a hormone inhibitor, I, it would be expected that the ternary complex (IRE) (depicted as HRE in Fig. 1) would be biologically inactive and that for the inhibitor, K_2 would equal K_3. A fourth equilibrium that can occur is the dissociation of the hormone-receptor-effector complex according to the equation:

$$H + RE \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} HRE \qquad K_4 \quad (4)$$

The above simple equilibria can be rendered more complex, for instance, by supposing that at least two hormone-receptor complexes must cluster before an effector molecule can interact, for example

$$2H + 2R \rightleftharpoons (HR)_2 \qquad K_5 \quad (5)$$

$$(HR)_2 + E \rightleftharpoons (HR)_2E \qquad K_6 \quad (6)$$

A more generalized scheme than the one outlined above has been described by DeHaen (24) for the action of hormones that stimulate adenylate cyclase.

It can readily be demonstrated from the above equilibria that:

$$K_3 \cdot K_4 = K_1 \cdot K_2 \qquad (7)$$

Given the above hypothesis, that $K_2 > K_3$ (i.e., the affinity of the receptor for the effector is greater in the presence of hormone), it is evident that $K_4 > K_1$. Thus the binding of a hormone by a homogeneous population of receptor molecules can, as suggested by the mobile receptor model, lead to complicated binding kinetics; the

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complicated binding kinetics predicted by the mobile receptor model (for example, nonlinear Scatchard plots) might otherwise (incorrectly) be interpreted in terms of a heterogeneity of receptor molecules.

The mobile receptor model introduces enormous flexibility into the possible actions of hormones and permits a complexity of kinetics that potentially may account for many observed phenomena in connection with hormone effects. It would, as suggested above and depicted in Figure 1, rationalize the differential modulation of independent membrane processes by a single hormone-receptor complex if the complex exhibited different affinities toward two or more membrane-localized effectors. Furthermore, as developed by DeHaen (24), the differential maximal activation of an effector (e.g., adenyl cyclase) common to several hormones could be explained in terms of distinct affinities of the various hormone receptors for the common enzymatic unit. Additionally, the complex *alpha-* and *beta*-adrenergic effects in biological systems may be explained in terms of the interactions of multiple receptors with multiple effectors.

An important aspect of the mobile receptor model concerns both the levels of effectors and receptors present in a given cell, and the receptor/effector ratio, which may well vary under different physiological conditions. For example, denervation supersensitivity might be rationalized in terms of variations in the receptor ratio. Alternatively, the ratio of adenyl cyclase effector molecules relative to the total number of receptors for distinct hormone activators would determine whether "additivity" of enzymatic activation might be observed for separate hormones.

The detailed mathematical analyses of the mobile receptor model by Jacobs and Cuatrecasas (55) and by DeHaen (24) indicate that the binding of a homogeneous ligand with a unique receptor molecule can exhibit nonlinear Scatchard plots, Hill plots consistent with "negative" cooperativity, *and* increased ligand off-rates determined with radiolabeled derivatives in the presence of high concentrations of hormone. By supposing a situation in which there is an excess of receptors (e.g., 10- to 20-fold) compared with effectors, the model can also predict the effect on the ligand binding data of "spare" but *equivalent* receptors. In such a case, at least two "affinity" sites for the ligand would be detected by binding studies, only one of which "sites" would appear to coincide with the ED50 for the biological dose-response curve.. All of the receptors would, nonetheless, be equivalent and would contribute to the overall responsiveness of the system.

Several studies have now confirmed that receptors for a number of active agents are mobile in the plane of the membrane. Fluorescent cholera toxin, which binds to membrane ganglioside, GM_1, and activates adenylate cyclase, can be observed to patch and cap in lymphocytes (20). Recently, studies with fluorescent derivatives of insulin and epidermal growth factor have confirmed that the receptors for these two polypeptides are also mobile (96, 102). Using image intensification photomicrography, the fluorescent hormones can be observed to bind initially in a diffuse pattern on the cell surface; subsequently the fluorescence can be observed first to aggregate in discrete patches and then to become internalized. The formation of receptor aggregates appears to depend on the presence of hormone. Strikingly, the internalization of both hormone-receptor complexes and ligand "acceptor" (see below) complexes appears to proceed via the same cell surface structures, the so-called "coated pits" (38, 39, 74a). The hormone-receptor complexes are remarkably mobile, as estimated by photobleaching recovery techniques, such that the microscopic aggregation of hormone-receptor oligomers could occur within milliseconds (95a).

C. Receptor Aggregation and Hormone Action

Given that the macroscopic aggregation of receptors can be visualized by fluores-

cence photomicrography, it is reasonable to anticipate that receptor aggregation at the molecular level may play a role in cellular activation. Studies with antibodies directed against the receptor for insulin appear to implicate receptor aggregation in insulin action (61). Remarkably, antibodies directed against the insulin receptor can be shown to possess insulin-like activity both in adipocytes and in muscle (57, 60, 61, 67a). However, monovalent antibodies, prepared by enzymatic digestion of the intact molecules, are not only devoid of insulin-like activity in adipocytes, but behave as competitive inhibitors of insulin binding and insulin action in these cells; furthermore, the monovalent human antibodies, once bound, can be rendered active in the presence of anti-human IgG, which would presumably cause crosslinking and aggregation of the monovalent antibody-receptor complex (61). Thus, for insulin and possibly for many other hormones, receptor clustering may prove to be a prerequisite for hormone action. In terms of the mobile receptor paradigm, the simplest initial situation may be the one outlined by equation 5, where two hormone-receptor complexes dimerize before interacting with an effector.

D. New Directions and the Mobile Receptor Model

The above discussion should serve to indicate that the mobile receptor model is sufficiently versatile to accommodate many of the phenomena observed in connection with hormone action. To date, the model has proved of considerable value in stimulating new experiments. The predicted mobility of receptors can now be visualized experimentally (96, 102). There is also now evidence that a receptor such as the one for insulin can interact with other nonrecognition macromolecules present in the plasma membrane (73, 74). It will thus be of interest in future work to determine the factors that control the mobility and clustering of hormone-receptor complexes and to characterize those membrane-localized macromolecules (possibly effectors) with which receptors can interact. It is to these ends that more recent studies are now being directed.

III. Receptors, Acceptors, and the Problem of Nonspecific Binding

In large part progress in the understanding of hormone-receptor interactions has been due to the development of reliable sensitive binding assays for the detection of pharmacological receptors. However, in binding studies it is often a problem to determine whether the site detected experimentally reflects a "true" pharmacological receptor. Critical to an interpretation of ligand binding data, therefore, is an understanding of the "receptor" concept, which has been so productive in analyzing, from a pharmacological point of view, the action of endogenous or foreign ligands (i.e., hormones, drugs) at the molecular, cellular, or supracellular level. The main aspects of the receptor concept, deriving directly from the work of Langley (65), Ehrlich (32), and Clark (16, 17), point out the importance of ligand-specific membrane-localized "receptive substances," present in vanishingly small numbers, that are critical in generating a biological response. The notion of biological responsiveness is thus inextricably linked to the notion of a cellular receptor. Therefore, information concerning the pharmacology of a particular ligand-responsive system of interest (e.g., for insulin-sensitive glucose transport in fat cells or *beta*-adrenergic stimulation of adenylate cyclase in a variety of membrane preparations) has permitted the development of certain operational criteria that are thought to typify hormone-receptor interactions. These criteria, having to do with appropriate ligand affinities, saturability, reversibility, stereochemical specificity, and appropriate tissue distribution are of enormous help in interpreting binding data for active ligands (51) and in discriminating "specific" (i.e., receptor-related) from "nonspecific" binding. Furthermore, it is becoming important to distinguish receptor-related binding from the binding of certain ligands

to specific cell surface components that may be termed "acceptors."

A. Receptors Versus Acceptors

It is becoming increasingly evident that the term "receptor" may require a more restricted and precise definition, given the ever burgeoning number of agents that are found to bind to the cell surface in a specific manner. In pharmacological terms, the membrane-receptor for agents such as neurotransmitters and hormones can be thought of as a macromolecule (probably an oligomer) that has the dual function of both recognizing a ligand of interest in a chemically specific manner (recognition function) and causing an immediate perturbation of membrane function (i.e., the action function) that in some manner leads to a biological response (for example, a cholinergic receptor-mediated change in membrane permeability leading to depolarization and consequently, muscle contraction). This recognition-action function of a receptor may be distinguished from a membrane acceptor site that may function solely as a recognition molecule for the selective cellular uptake of certain serum-borne constituents.

An example of a cellular "acceptor" can be seen in the function of transcobalamin II (TCII), a molecule that serves as a transport protein for vitamin B_{12} (cobalamin) in the circulation and subsequently delivers cobalamin to the cell interior (71, 77, 78, 100). In the case of cobalamin, the TCII-cobalamin complex can bind to a specific cellular acceptor site, resulting in the translocation of the complex and the intracellular release of cobalamin for further metabolic processes. In this instance, cobalamin can be thought of as the pharmacological agent active at an intracellular (enzyme) receptor; the membrane constituent that recognizes the TCII-cobalamin, in a highly specific manner (for example, the TCII-cobalamin complex is bound much more tightly than either free TCII or free cobalamin) clearly functions in a manner different from the one envisioned for hormone receptors, and may, therefore, be termed an "acceptor." The cellular binding site for low-density lipoprotein (LDL) (1a, 38, 39) can be thought of in similar terms, where the feedback regulator cholesterol is the pharmacologically active ligand in the cell interior after internalization via the LDL acceptor. For acceptors, it is expected that the chemical specificity of recognition will be just as stringent as is the case for receptors, such that nonspecific binding artifacts can be readily recognized. Additionally, based on the observations of an apparently common site of internalization of α-2-macroglobulin (74a, 116), LDL (1a), and epidermal growth factor-urogastrone (EGF/URO) (the "coated pit"), one can predict that there may be common mechanisms controlling the mobilities and some of the membrane-localized interactions of both receptors and acceptors. Indeed, one can also predict further that there may be structural elements common among all receptors and acceptors. Nonetheless, it is important to make a clear-cut distinction between receptors and "acceptors" since a major challenge for further work with receptors is to define the portion of the recognition molecule that is related to cellular stimulation. Thus, in this review, the term receptor is used in a restricted sense to designate a recognition-action macromolecule.

IV. Receptor Regulation

A most exciting aspect of cellular control that is being intensively explored with the use of ligand binding techniques has to do with the regulation of cell receptors. As indicated above, increasing numbers of studies now demonstrate that, rather than being relatively static components of the plasma membrane, receptors are in a dynamic state of turnover under the influence of a variety of factors. In addition to a preprogrammed rate of synthesis and turnover, cell receptor numbers can be observed to change with the stage of the cell cycle, with the state of cellular development and differentiation and under the influence of exogenous ligands including both receptor-

specific hormones (i.e., homospecific down- or up-regulation) as well as receptor-nonspecific hormones and other compounds (i.e., heterospecific down- or up-regulation). In the following paragraphs, some selected illustrative examples of these aspects of receptor regulation will be given.

A. Receptor Biosynthesis

Enormous strides have been made in the study of the "nicotinic" receptor for acetylcholine in a variety of tissues, in large part due to the availability of the potent selective irreversible receptor-blocking agent, α-bungarotoxin [see review by Karlin (63)]. In cultured chick skeletal muscle cells, measurements have revealed a relatively rapid incorporation of newly-synthesized intracellular cholinergic receptors into the plasma membrane (29–31, 43). It can be observed that after the muscle cultures are switched from regular culture medium to a medium containing ^{2}H-, ^{13}C-, and ^{15}N-amino acids, the rapidly labeled "heavy receptors" appear in the plasma membrane within a 3- to 3.5-hour period. Under these conditions of culture, the precursor-product relationship between the pool of intracellular receptors and surface receptors lies somewhere between a strictly linear assembly-line process and a random process of selection of receptors leading from the intracellular pool to the cell surface. It is likely that the dynamics of the cholinergic receptor in the cultured chick muscle cells (29–31, 43) will reflect the dynamics of other membrane-localized hormone receptors that, like the cholinergic receptor, are known to be glycoproteins. The use of the elegant "heavy receptor" technique referenced above (31) and of highly selective irreversible receptor-labeling reagents should prove of utmost value for similar studies with other receptors.

Aside from the ongoing process of receptor synthesis and insertion, there is the question of whether de novo synthesis will in fact take place. At least two interesting examples of the all-or-none aspect of receptor synthesis can be cited. In lymphocytes, a membrane-localized binding site for insulin, probably related to cellular cytotoxic activity (46, 108) can be detected after, but not before, cellular activation by a variety of natural and artificial stimuli (46, 50, 64). Before stimulation by plant lectins, neither cell surface nor cytoplasmic binding sites for insulin can be detected; in step with the onset of DNA synthesis and the appearance of mitogen-induced blast cell transformation, there is an appearance of binding sites for insulin. Somewhat surprisingly, neither cell division (50) nor DNA synthesis per se (45, 46) appear to be prerequisites for the appearance of the lymphocyte insulin binding sites. It is concluded that under the above circumstances, insulin receptors appear de novo, dependent on the state of differentiation of the lymphocyte induced by the mitogenic stimulus (either lectin, lipopolysaccharide, periodate oxidation, or miss-match of a histocompatibility locus). The appearance of receptors for insulin is to be distinguished from the insulin-modulated variation in insulin binding by monocytes (99; also see below).

B. Developmental Aspects of Receptor Regulation

A second instance of "programmed" receptor variation can be observed for β-nerve growth factor (β-NGF) in the course of chick development (48). Whereas early in development (8–14 days) chick dorsal root ganglia are highly responsive (neurite outgrowth) to β-NGF, further on in development (18 days and thereafter) no response is observed; the binding of ^{125}I-labeled β-NGF to the ganglion cells broadly parallels responsiveness, in that appreciable binding is detected at day 8, rising to a maximum at about day 14, and dropping off to comparatively very low levels by day 21. It is to be noted that ligand responsiveness and ligand binding capacity need not necessarily go *pari passu*. For example, in the case of the *beta*-adrenergic receptor in rat red blood cells, as the reticulocyte develops into the mature erythrocyte, there is a marked reduction in isoproterenol respon-

siveness (adenylate cyclase) without a concomitant reduction in the number of catecholamine receptors (15). The change in responsiveness can be attributed to changes in the coupling between receptor occupation and enzyme activation (4, 7, 8).

C. Growth Control and Receptor Regulation

In addition to a programmed control of receptor density, the stage of the cell cycle in actively growing cells is thought to play a role. In cultured mouse Balb/3T3 fibroblasts, the maximum binding of insulin by rapidly growing cells can be observed to be lower than that bound by cells that stop growing either as monolayer confluency is reached or under the influence of a reduced serum concentration (111). In contrast, cells at low-density in culture may possess up to eight times as many receptors for epidermal growth factor-urogastrone (EGF/URO) as do cells at confluency (52).

D. Hormonal Modulation of Receptors: Homospecific versus Heterospecific

As indicated above, the ability of hormones to regulate either their own (homospecific) or other (heterospecific) receptors is a new area of considerable interest. As summarized in a review by Raff (86), comparatively early work with cell surface antigens and antibodies dating back to the late 1950s, demonstrated the selective removal from the cell surface of "ligand-receptor" complexes. A stimulus for the examination of an analogous process for hormone receptors came from the work of Gavin et al. (33), who observed a reduction of binding sites for insulin in cultured IM-9 lymphocytes that had been preexposed to comparatively high (relative to physiological concentrations) concentrations of insulin. While the mechanism and insulin-specificity of the "down-regulation" observed in the initial studies has been questioned (53), subsequent work with other hormones has yielded compelling evidence that preexposure of an intact cell to a particular hormone can selectively reduce the subsequent binding of the labeled ligand.

It is, however, important to sound a cautionary note for all studies of homospecific receptor regulation. As in the case of the receptor binding studies themselves, it is necessary to demonstrate that the observed down-regulation is truly ligand-specific. This appears not to be entirely so for insulin, in which case there can be a reduction of receptor number unrelated to receptor occupation (33, 53), as well as a small but detectable reduction in binding sites for hormones other than insulin (53; also see Fig. 5, ref. 68). The use of *beta*-adrenergic agonist analogues to reduce the number of binding sites for ^{3}H-labeled alprenolol in cultured frog erythrocytes in proportion to the known potencies of such compounds in *beta*-adrenergic systems (i.e., isoproterenol > epinephrine > norepinephrine) provided more convincing data concerning receptor homoregulation (75). Strikingly, from studies of the *beta*-receptor, it appears that antagonists, in contrast to agonists, are unable to regulate receptor number; simple receptor occupation does not appear to be sufficient to bring about regulation (67). In addition to the above concerns, as with binding studies, it is important to compare the reduced binding with some alteration in ligand responsiveness. Provided that there are not receptors far in excess of those required for a maximum response (i.e., provided there are no "spare" receptors), homospecific down-regulation should be accompanied by a reduction in the maximum biological response, without a change in the concentration of effector causing an effect 50% of maximum. A result of this nature has been suggested for the homoregulation of luteinizing hormone receptors linked to the stimulation of adenylate cyclase in rat ovary membranes (19). In the case where a large number of spare receptors are present, it can be predicted that a significant reduction of available binding sites to a level still higher than that initially required for a maximum response should shift the dose-response curve to the right (i.e., more hormone will be required in the external medium to occupy the desired number of receptors of identical affinity) but should not

affect the maximum response attained. In such experiments, it is important to demonstrate that response to the homospecifically down-regulated receptor is altered, while the same response (e.g., adenylate cyclase or muscle contraction) to another hormone is unaltered. It should be noted that homospecific regulation of a receptor can be in the positive as well as negative direction, as indicated by the studies with prolactin, which can induce the appearance of prolactin receptors in the liver of hypophysectomized rats (84, 85). Another striking example of the upward regulation of receptor comes from studies with angiotensin II, which upon administration in rats causes initially (24 h) an increase both in receptor number and receptor affinity but subsequently (36 h) leads only to an increase in receptor number; the response in vitro of the target adrenal cells isolated from the zona glomerulosa is increased in step with the increase in receptor number (1, 43a). It is important to point out that the results demonstrating an upward regulation of receptors have been obtained in whole animals; it is, therefore, not clear whether the mechanism for the upward regulation resides at the level of the receptors on the target cells that are affected or whether an indirect mechanism via a receptor at a remote site is involved. Presumably, studies of responsive cells exposed to various ligands in vitro will resolve this question.

From the above discussion, it should be apparent that criteria as stringent as those used to distinguish receptor-related from nonspecific binding should also be employed in studies aimed at demonstrating homospecific receptor regulation. Further, in studies of homospecific down-regulation, it is important to determine whether the receptor has actually disappeared (e.g., by internalization) or whether the receptor is still present in the plasma membrane, perhaps in a cryptic form.

It is also the case that one hormone can regulate receptors for another hormone—so-called heterospecific receptor regulation. For example, in rat ovarian fragments (but not the isolated, cultured granulosa cells) follicle stimulating hormone (FSH) causes the appearance of specific binding sites for ^{125}I-labeled human chorionic gonadotropin (presumably sites intended for luteinizing hormone) (79). In addition to polypeptides regulating receptors for other polypeptides, it is undoubtedly the case that nonpeptide hormones will be found to regulate receptors for polypeptide hormones, and vice versa. Specific examples can already be cited, such as the ability of estrogen, under selected conditions, to induce the appearance of receptors for oxytocin in rat uterus (107) and the appearance of receptors for both prolactin and growth hormone in rat liver (84). Additionally, the numbers of fibroblast receptors for EGF/URO can be increased under the influence of corticosteroids (3).

E. Receptor Regulation and Tachyphylaxis

A long-recognized pharmacological phenomenon concerns the diminution in response to a system upon repeated exposure to an agonist: so-called tachyphylaxis or desensitization. In many cases the phenomenon is specific, in that the response (for example, muscle contraction) to one agent is markedly diminished, whereas the *same* response to a second agent is unaffected. The receptor-related mechanisms for tachyphylaxis are as yet poorly understood. Nonetheless, the down-regulation of receptors observed by ligand binding studies provides one possible mechanism. Both the reduction in ligand binding and tachyphylaxis appear to be caused by agonists, but not antagonists. It is as yet unclear whether receptors are selectively lost from responsive cells, for example, by shedding or by internalization, or whether the receptor remains inaccessible in the membrane as a high-affinity hormone receptor complex. In the case of the nicotinic receptor for acetylcholine, it is believed that the "high-affinity" form of the receptor that can be detected in detergent extracts of electroplax membranes represents the desensitized form of the receptor (115). In view of this

possibility, a further equilibrium might be considered in terms of the mobile receptor paradigm discussed above:

$$\mathrm{HRE} \rightleftharpoons \mathrm{HR'} + \mathrm{E} \quad (7)$$

$$\mathrm{HR'} \rightleftharpoons \mathrm{HR} \quad (8)$$

where HR′ represents an altered high affinity state of the hormone receptor complex that might be produced only consequent to the formation of the complex, HRE. In a sense, the agonist, in terms of the rate theory of drug action (80a) becomes its own ideal antagonist, with a markedly reduced off-rate. A conformational change of the kind outlined by equations 7 and 8 has often been suggested as a mechanism for desensitization, as discussed by Colquhoun and Rang (18).

A provocative example of receptor desensitization comes from work with angiotensin analogues that are full agonists in causing ileal contraction but, unlike native angiotensin II, do not lead to desensitization of the preparation (81). Thus, the conformation of the hormone-receptor complex that leads to cellular activation may differ from the conformation that leads to receptor-specific desensitization (or down-regulation). It is evident that the agonist property of compounds may be a necessary but not sufficient condition for the production of tachyphylaxis. It will thus be of interest to ascertain the role of receptor mobility in connection with the phenomenon of desensitization.

F. Nonspecific Factors That Affect Receptors

An aspect of receptor regulation that cannot be overlooked concerns the ability of agents other than hormones to regulate receptor density in cells. For instance, butyric acid causes an induction of *beta*-adrenergic receptors in cultured HeLa cells (110), while cyclic AMP increases the concentration of insulin receptors in cultured fibroblasts and lymphocytes (111). Consequent to viral transformation, there can be a marked reduction in receptors, as is seen for EGF/URO receptors in cells transformed by murine or feline sarcoma viruses (113); an analogous reduction in receptors for EGF/URO can be observed consequent to chemical transformation of cultured hamster fibroblasts (49). Strikingly, viral transformation may change not only the number of receptors present, but can also change the receptor recognition property, as is seen in the change from β_{-1} to β_{-2} selectivity of the catecholamine receptors in cultured mouse 3T3 cells consequent to transformation with simian virus 40 (103). Thus, the number of receptors present at the cell surface can be affected by a large number of factors both secondary to internal cellular events (rates of synthesis and turnover, cell cycle, cell differentiation) and consequent to a variety of external stimuli caused by hormones and other agents, either related to hormone receptor occupation (homospecific or heterospecific regulation) or to other cellular effectors (for example, viruses, chemicals, or other toxins).

V. Ligand Internalization and Hormone Action

There is now incontrovertible evidence that the cell membrane constitutes the primary site of action for many hormones including polypeptides, catecholamines, cholinergic compounds, and other neurotransmitter substances. A reasonable question to pose, however, is: Does the cell surface constitute the only site of action of such hormones? Recent ligand binding studies with radioactively labeled polypeptides are directing a close look at this question. It is now evident from a number of studies that, subsequent to the binding of a radioactively labeled substance at the receptor site, ligand internalization can occur. In the case of mouse EGF/URO, the disappearance in intact cells of available receptor sites (apparent "down-regulation") observed consequent to the binding of ligand (2,13) is associated with the appearance in the medium (at 37°C) of ligand degradation products (13). It appears that

the EGF/URO-receptor complex once formed can undergo pinocytosis and lysosomal degradation. As alluded to above, such a mechanism is thought to liberate cobalamin into the cell interior from TCII-cobalamin complex and cholesterol from the LDL-cholesterol complex. It is, therefore, not unreasonable to hypothesize that a degradation fragment of EGF/URO itself or possibly of the receptor, released in the cell interior subsequent to receptor binding and internalization, may play a role in the well known mitogenic action of this polypeptide (however, see discussion below). Since it is the rule, rather than the exception, that at least some proportion of an active ligand present in the external medium can be found in the cellular cytoplasm, the proof or disproof of the above hypothesis may be much more difficult than it was to establish the cell surface as the primary point of hormone action. Even in the case of well-controlled studies with ligand-agarose derivatives, it could be argued that a portion of the ligand might be cleaved at the cell surface so as to permit selective internalization without the release of appreciable ligand into the medium.

The above arguments are not to be construed as opposing the main tenet that has developed, implicating membrane localized reactions in the action of perhaps the majority of neurotransmitters and related hormones. Indeed, one might predict that all agents bringing about rapid (i.e., seconds to minutes) cellular events (e.g., membrane depolarization, stimulation of glucose transport, activation of adenylate cyclase, and modulation of cyclic AMP-dependent processes) would act solely at the plasma membrane. In this context, the process of ligand internalization and lysosomal degradation may prove to be an important aspect of receptor regulation per se that is linked to but separate from the "action function" of the receptor, as envisioned by the mobile receptor paradigm discussed above. In such cases, receptor internalization may function to degrade receptors (perhaps as part of a "clearing" process) and thus decrease their surface density. The observation that anti-insulin receptor antibodies exhibit insulin-like actions in isolated adipocytes (57,60,61) argues convincingly, if not unequivocally, in favor of a membrane-localized site of action for insulin. Furthermore, the stimulatory action of the antibodies suggests that the information for cell activation resides in the receptor itself and not in the insulin molecule.

It is important, nonetheless, to consider seriously the possibility that ligand-receptor internalization may play a role for agents that cause a relatively slow (hours to days) cellular response such as cell division or nerve cell differentiation. For example, insulin, which can cause both rapid (glucose transport, antilipolysis) and delayed (fibroblast growth) cellular effects, could potentially act both at the cell surface and via an internalization process, depending on the cell type affected. The detection of nuclear binding sites for insulin (35–37), while controversial (6), is of interest in this regard.

To date, the best data related to polypeptide action and ligand internalization come from studies with mouse EGF/URO, an agent that can also cause both rapid (inhibition of gastric acid secretion) and delayed (cell growth) cellular effects. As mentioned above, highly fluorescent analogues of both EGF/URO and insulin can be observed to bind initially to highly mobile receptor sites in viable fibroblasts (42,96,102); subsequently, the receptors can be observed to cluster rapidly in small discrete extracellular patches, which within 10 minutes at 37°C become internalized. Other experiments using antibodies to monitor the disappearance of cell surface EGF/URO (13), using ^{125}I-labeled EGF/URO to monitor the "down-regulation" of the receptor (2) and using photoaffinity-labeled EGF/URO receptor to follow receptor processing (23a) document further the internalization of the EGF/URO-receptor complex.

The relationship of EGF/URO receptor internalization to cellular stimulation is as yet uncertain. Strikingly, the addition of

anti-EGF/URO antibody to cells stimulated by EGF/URO can reverse the mitogenic response as long as 6 to 8 hours after the initial stimulus (13,101). At such a time, a large proportion of receptors would have become internalized and degraded so as to liberate fragments both of the receptor and the polypeptide in the cytoplasm. Further, it can be demonstrated that a very brief exposure to EGF/URO (30 min) followed by removal of EGF/URO from the medium is sufficient to trigger a mitogenic response (101). Taken together, the above data make it difficult to attribute the mitogenic process to internalized receptor or hormone fragments. Clearly, further work will be necessary to clarify the role of receptor internalization (and shedding) in the stimulation of cells by hormones like EGF/URO and insulin.

VI. Receptor Cooperativity and Hormone Action

It is often the case that an analysis of ligand binding data, (for example, by the method of Scatchard (95), suggests either the presence of ligand-receptor cooperativity or the presence of more than one class of ligand binding sites. While there are a number of possible factors that can result in nonlinear Scatchard plots of the data [discussed at some length by Rodbard (87) and Rodbard and Bertino (88)], recent discussions in the literature have favored a negative cooperativity model for the interaction of a number of hormones with specific receptors. The interpretations rest on two principal kinds of data: 1) equilibrium binding data yielding Scatchard plots that are concave up; and 2) a kinetic analysis of ligand-receptor dissociation kinetics done either in the absence or presence of an excess of unlabeled ligand.

It should be noted at the outset that the interpretation of both kinds of data is fraught with difficulty. Firstly, even if the equilibrium binding data are interpreted in terms of multiple binding sites, it is often very difficult to establish the ligand specificity of each binding site according to the criteria outlined above and discussed elsewhere (51). Furthermore, the occurrence of anomalous dissociation kinetics in nonreceptor preparations such as talc (22) indicates that the unequivocal analysis of similar data in biological systems may prove difficult. As originally demonstrated, the talc binding data for insulin can be seen to be of a nonreceptor character, distinct from data obtained in membrane preparations.

The most extensively documented data supporting a negative cooperativity model come from work with insulin (26–28a). Based primarily on an assay measuring the accelerated dissociation rate of ^{125}I-insulin from cultured IM-9 lymphocytes and mouse liver membranes caused by a variety of insulin analogues, it has been concluded that the insulin molecule possesses a receptor binding region as well as a distinct region responsible for causing an increased insulin receptor dissociation rate (28a). Despite these detailed studies with insulin analogues that are consistent with an agonist-mediated acceleration of the dissociation of previously bound hormone, a careful kinetic analysis of insulin binding to either unsaturated or partially saturated receptor preparations reveals no difference in the intrinsic receptor affinity constant for insulin (83); the data of Pollet et al. (83) appear to be incompatible with negative cooperativity. It is evident that different methods of kinetic analysis yield apparently conflicting results. The kinetic experiments of both DeMeyts and coworkers (26–28a) and Pollet et al. (83) have been recently reevaluated by DeLean and Rodbard (25) so as to indicate that computer-simulation of a model for cooperative binding reproduces neither the data of Pollet et al. nor those of DeMeyts and coworkers. The discussion of DeLean and Rodbard (25) suggests further experiments to evaluate the insulin binding kinetics in cell and membrane systems. It is significant nonetheless that the equilibrium binding data (concave-up Scatchard plots) are consistent with a complex process of insulin binding by the receptor. It should be evident from

the above discussion that, in the absence of evidence at least as extensive as that obtained by DeMeyts and coworkers (26–28a) for insulin, the simple demonstration that a ligand enhances its own dissociation rate is insufficient to confirm or disprove receptor cooperativity.

A consideration of insulin action in terms of the mobile or floating receptor hypothesis described above predicts that insulin binding by a single receptor macromolecule could readily exhibit multiple affinities, as well as negative cooperativity (55). Recent work suggests that, whereas the soluble insulin receptor isolated from rat liver and placenta membranes by affinity chromatography does not exhibit negative cooperativity, other membrane-localized constituents can interact with the insulin receptor, so as to increase the receptor's apparent molecular size and to confer upon the receptor the complicated equilibrium binding kinetics observed either in membrane preparations or in crude soluble receptor preparations (73,74).

Thus, while the receptor binding of insulin may indeed exhibit cooperativity, as elaborated upon by DeMeyts and coworkers (26–28a), a model akin to the mobile receptor paradigm, wherein receptor-effector interactions lead to alterations in ligand affinity is suggested, rather than a model comprising site-site interactions between receptors, as was originally proposed. Future work should provide data to distinguish between the models not only for insulin, but also for other hormones for which cooperative receptor interactions are suspected. It should be noted that the role of receptor cooperativity in terms of biological response is readily accommodated by the mobile receptor model. Additionally, the mobile receptor model can accommodate observations concerned with the binding of cholinergic agents to the muscarinic receptor (9), where the data suggest that agonists (which would promote receptor-effector interactions) possess at least two receptor affinities, whereas antagonist binding appears simple; differences in agonist and antagonist binding have also been observed for a variety of other active ligands (see reviews 9 and 106). It is thus possible that a multistate (possibly cooperative) receptor model may prove to be the rule, rather than the exception, for the action of a large number of neurotransmitters and hormones.

VII. Hormonal Modulation of Adenylate Cyclase

The coupling mechanism, whereby receptor occupation leads to cellular activation, has been studied most thoroughly for those hormones that act via cyclic adenosine monophosphate, subsequent to the stimulation of membrane-localized adenylate cyclase. Indeed, this aspect of study is in such an active state that the following can be considered only as an interim progress report.

A. Distinction of the Ligand Recognition Site from the Enzymatic Catalytic Site

Although the receptors for those agents activating the cyclase appear to be intimately linked to the enzymatic activity (58,76,89,109), evidence from a number of sources now convincingly demonstrates that the ligand recognition site and the enzyme converting ATP to cyclic AMP reside in distinct molecular species. Part of the evidence is inferrential, based on a consideration of the number of independently acting agents that can activate adenylate cyclase in a single cell such as the adipocyte (21,24). Other more direct evidence comes from: 1) cell fusion experiments (80,97,98) wherein a receptor-deficient cell possessing an active adenylate cyclase can be rendered responsive to a hormone subsequent to fusion with a receptor-containing cell in which the cyclase has been inactivated by N-ethylmaleimide; 2) studies of cellular differentiation, in which there are nonparallel changes in the number of *beta*-adrenergic receptors, fluoride-sensitive cyclase, and catecholamine-sensitive cyclase. This nonparallelism can be observed during rat erythrocyte maturation (4,7,8,15,62) as well as in cultured HeLa cells subsequent to the

induction of *beta*-receptors by butyrate (47); 3) studies of membrane fractionation subsequent to cellular disruption, in which the *beta*-receptor and adenylate cyclase can be observed to partition with different distributions in sucrose density gradients (93); 4) solubilization and chromatographic separation of the adenylate cyclase and the *beta*-adrenergic receptor (40,70); and 5) genetic evidence (12,41,54) suggesting that the *beta*-adrenergic receptor and adenylate cyclase are products of separate genes.

A very exiciting aspect of the genetic studies with S-49 lymphoma cell lines was the discovery of a mutant possessing both a *beta*-adrenergic recognition site and a functional adenylate cyclase, but in which cell receptor occupation does not lead to cyclase activation. The discovery of the "uncoupled" cells (so-called UNC mutant) (41) has stimulated work to isolate the "coupling factor," thought to be a protein responsible for the guanosine triphosphate-mediated modulation of hormone-stimulated activation of adenylate cyclase. Not only does the coupling of receptor activation to cyclase activation appear to require a protein mediator, but the catalytic activity per se [for example, activity stimulated by sodium fluoride and guanylyl-5′-imidodiphosphate (GPP(NH)P)] may be modulated by one or more membrane-localized protein factors (44,94). Recent new advances in the assay and detection of regulatory proteins in this complex system indicate the existence of separable components that can be, at least in part, reconstituted (14,44,82,90,91,94). As suggested recently (91), the receptor-cyclase complex may comprise at least three regulatory proteins in addition to the catalytic and ligand recognition moieties. Clearly, the picture concerning the hormonal modulation of adenylate cyclase activity and the nonhormonal modulation of this enzyme, which is central to the action of a variety of neurotransmitters, hormones, and toxins is changing and expanding at a rapid pace. Many new developments in this area are anticipated in the near future.

B. On the Question of Receptor-Adenylate Cyclase Coupling

As described above, there is now unequivocal evidence that hormone receptors and adenylate cyclase are separate macromolecules whose function and physical state can be regulated and monitored independently, as predicted by the mobile receptor hypothesis. However, it must be acknowledged that there is presently no evidence that receptors and cyclase molecules are physically associated, even in the presence of hormones. It is possible that, as suggested by Tolkovsky and Levitzki (114), the intermediate, HRE, may have a very short half-life and may never accumulate as such. Nonetheless, now that these molecules have been proved to exist apart, positive proof must be provided in order to conclude that functional coupling (i.e., modification of enzyme activity) is related to direct physical coupling; the latter has been nearly universally assumed to occur, even in the "collision coupling" version (114) of the mobile receptor hypothesis. It is perhaps ironic that, whereas early studies of the hormone-cyclase system have led to this "mobile receptor model," more recent data are suggestive that alternative models may be necessary.

In the absence of evidence for direct receptor-effector (cyclase) association, the possibility of indirect coupling must be seriously entertained. In fact, it was recently speculated on the basis that adrenergic receptors and cyclase can be found in different vesicle populations derived from erythrocyte ghosts (even when prepared in the presence of an agonist, isoproterenol), that the hormonal regulation of this enzyme could in principle be mediated indirectly (93). In such a case, cyclic AMP would very possibly not be literally the true "second" messenger of hormone action. Also, although not interpreted in this manner by the authors, the kinetics of coupling of hormonal reconstitution in cell fusion experiments (97) may be too rapid to be explained simply on the basis of protein intermixing

to allow direct receptor-cyclase associations; the rate of the process of reconstitution appears to be almost coincident with that of the rate of fusion. Possible mechanisms by which hormone-receptor complexes could affect adenylate cyclase, other than by direct association, include intermediary, primary, hypothetical chemical substances (for example, resulting from membrane phospholipase activation), changes in ionic caryonition, and/or electrochemical gradients across the membrane, or through an interconnecting network of a submembranous protein mesh that may simultaneously alter distant proteins.

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