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# COMMENTARY

## INSULIN RECEPTORS, CELL MEMBRANES AND HORMONE ACTION

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DURING the past few years, there has been a large burst of interest in membrane receptors for hormones, and considerable progress has been made in the identification and study of receptors for such peptide hormones as insulin, glucagon, adrenocorticotropin, thyrotropin, angiotensin, calcitonin, growth hormone, prolactin, follicle-stimulating hormone, leutinizing hormone, chorionic gonadotropin, oxytocin and vasopressin, as well as nonpeptide hormones such as catecholamines, prostaglandins and acetylcholine (reviewed in Ref. 1). The general approach in these studies has been to measure the interaction (binding) of a radioactively labeled hormone with intact target cells or with isolated membrane preparations derived from such cells. The binding is surmised to reflect "specific" receptor interactions if it demonstrates: (a) strict structural and steric specificity; (b) saturability, which indicates a finite and limited number of binding sites; (c) tissue specificity in accord with biological target cell sensitivity; (d) high affinity, in harmony with the physiological concentrations of the hormone; and (e) reversibility, which is kinetically consistent with the reversal of the physiological effects observed upon removal of the hormone from the medium.

A number of problems and pitfalls can be encountered in the kinds of studies described above, and considerable caution must be exercised in interpretation of data (reviewed in Refs. 1 and 2). Because physiological membrane receptors are present in extremely small quantity in membranes, the hormone must be labeled to very high specific activity (e.g. with $^{125}I$ or $^{131}I$ at 1–2 Ci/$\mu$mole) without destroying the biological activity of the hormone. Since virtually all chemical compounds used as binding ligands (hormones) exhibit some nonspecific adsorptive or binding properties to a variety of inert as well as nonreceptor biological materials, since such "binding" may be of extremely high affinity, and since the number ("infinite", by definition) of such nonspecific binding sites greatly exceeds that of specific receptors, great difficulties may be encountered in detecting such specific receptors. These problems may be compounded by the fact that numerous kinds of heterogenous nonspecific binding sites (differing in affinity) may coexist, the sum of which can for example result in Scatchard plots which, at high concentrations of bound ligand, give the appearance of a "second" class of receptors. Furthermore, if nonspecific adsorptive materials are present in very small quantity, binding to these materials can exhibit saturability and

even stereospecificity; for example, such saturable binding has been observed with peptide hormones to certain kinds of filters and to glass materials, and the differential binding of D- and L-stereoisomers of tryptophan to albumin is well known. In addition, cells and biological membranes may contain "specific" functional structures (e.g. degradative or metabolizing enzymes) which are strictly "nonspecific" with respect to receptor interactions but which may masquerade as receptors because they satisfy many of the binding criteria described above.

The binding data (specificity, affinity, number of sites, reversibility) must therefore be scrupulously evaluated by careful and detailed comparisons with the biological activity of the hormone. It is desirable that the initial preparation used for binding studies be a simple, intact system (e.g. isolated, homogeneous and viable cells) so that binding and biological responses can be measured in the same system, before disruptive procedures are performed on the cells.

In some systems, such as with insulin, glucagon and some other peptide hormones, it is reasonably certain that specific receptor interactions can be detected with $^{125}$I-labeled hormones. The potential perils of using binding studies to detect biological receptors can be illustrated with the case of the catecholamine hormones. The binding of $^{3}$H-labeled (5–10 Ci/m-mole) catecholamines (norepinephrine, isoproterenol) to various mammalian cells and membrane preparations shows saturability, an apparent affinity not too dissimilar from that which is presumed to operate for the intact receptor system, and specificity for the catechol moiety of the ligand.[3,4] However, close scrutiny demonstrates that the binding is totally lacking in stereospecificity, that the ethanolamine portion of the molecule is not required for binding (e.g., pyrocatechol is as good as (−)-norepinephrine as a competing ligand), that the binding is essentially irreversible, and that the number of binding sites, compared to the corresponding number for peptide hormones, is enormously high.[4] In addition, it has been established that catechol substances (e.g. pyrocatechol, 3,4-dihydroxymandelic acid, the (+)-isomer of norepinephrine) which compete for binding indistinguishably from the labeled hormone are themselves biologically inert, and they do not inhibit either the biological effects of the active hormone in intact cells or the stimulation of adenylate cyclase activity in membrane preparations.[4] Furthermore, the non-catechol, *m*-methanesulfonamide derivative of (−)-isoproterenol, which is a potent $\beta$-adrenergic agonist, does not compete for binding. It is evident that the measured membrane binding of $^{3}$H-norepinephrine cannot represent direct interactions with $\beta$-adrenergic receptors. Some evidence has been presented[4] which suggests that the binding measured with $^{3}$H-norepinephrine represents binding to an altered form of a membrane-bound enzyme, catechol-*O*-methyl transferase. The number of true adrenergic receptors in these tissues must be very small, since in the presence of a large excess of pyrocatechol (or other catechols) the residual binding of $^{3}$H-(−)-norepinephrine is extremely small and it is still not stereospecific. Thus, this is an example of a "specific" but nonreceptor membrane-localized component which is present in very large excess compared to the true receptor components, and which because of some cross-specificity with the radioligand used, feigns a receptor structure. Detection of specific $\beta$-adrenergic receptors will probably require the use of binding ligands of much higher specific activity which either lack a 3,4-dihydroxy phenolic group or which must be used together with substances that suppress the non-receptor catechol-binding components.

The binding of insulin to a variety of cells and membranes has been studied in detail in various laboratories (reviewed in Refs. 1, 5 and 6). The binding observed is highly specific for this hormone, and chemical derivatives compete for binding in accord with their relative effectiveness as agonists. A saturable binding site of high affinity (about $10^{-10}$ M) can be discerned in fat cells, a value which correlates well with the known biological effects of the hormone in the same cells. Fat cells have very few receptors for insulin, about 10,000/cell or about $10/\mu m^2$ of surface area. The binding is spontaneously reversible, and the rates of dissociation and association can be measured independently. The insulin molecules which dissociate are presumably chemically intact, since their biological potency is unaltered. Thus, interaction of this hormone with receptors does not involve stable covalent bonds, and it is not accompanied by degradation or inactivation of the hormone.

These data suggest that receptor occupancy and activation by insulin may be simply dictated by the concentration of the hormone in the extracellular spaces, equilibration occurring at rates dependent primarily on the rate of dissociation of the hormone–receptor complex. It is not known, however, whether special hormone-specific mechanisms exist in peripheral tissues for internalizing or otherwise degrading insulin. Although insulin can be degraded by microsomal preparations and soluble enzymes from virtually all peripheral tissues, it is not known whether this degradation plays a significant physiological role in the normal disposition of the hormone in any tissue except the liver. Although the liver is clearly a key site for the normal degradative process, the mechanisms by which this process recognizes, fixes and degrades the hormone present in the circulation are presently obscure. Does the degradative system in the liver simply respond to the concentration of hormone in the medium? Are there chemical changes in insulin molecules in the circulation which, however subtle, earmark these molecules for degradation while the others escape? Could, for example, binding to specific serum proteins or specific enzymatic modifications (e.g. glycosylation) of the hormone provide a chemical basis for designating certain molecules for degradation? It is now known, for example, that the liver contains highly specific uptake (degradative) membrane sites which can recognize subtle changes in certain plasma glycoproteins, such as the exposure of a terminal galactose by the removal of a single sialic acid residue.[7] Such changes may not be accompanied by changes in the intrinsic biological activity of the protein. The answers to these and related questions may have important consequences in our understanding of normal insulin homeostasis, as well as of pathologic conditions such as those characterized, for example, by hyperinsulinism or insulin resistance.

The insulin-binding sites of fat cells are probably localized exclusively on the external surface of these cells, since virtually no binding is detected in the total particulate fraction obtained from cells previously digested with trypsin–agarose. Localization to the external aspect of the cytoplasmic membrane has been established by using inside-out membrane vesicles. Trypsin digestion of these vesicles does not destroy the binding which is detected upon subsequent rupture of the vesicles. If $^{125}$I-insulin is bound to the intact cell before endovesiculation and preparation of the inside-out vesicles, the hormone cannot dissociate unless the vesicles are disrupted physically or by detergent or phospholipase treatment. These studies also indicate that very little or no "flip-flop" of these receptors occurs between the outer and inner aspects of the membrane.

The receptor structures for insulin are probably glycoprotein in nature, since sequential digestion with neuraminidase followed by β-galactosidase decreases the affinity of the insulin–receptor complex, and since certain plant lectins appear to bind directly to these structures. These binding sites are very susceptible to proteases; mild digestion affects primarily the affinity of binding, whereas more extensive digestion appears to cause more drastic destruction of the binding function. One of the most interesting but unexplained features is the unmasking of considerable quantities of "new" binding sites by digesting cells or membranes with certain phospholipases or by exposure to high concentrations (2 N) of NaCl. The possible physiologic role of these cryptic sites is not understood.

Insulin-binding macromolecules have been solubilized from cell membranes with non-ionic detergents, and the binding properties of the solubilized material are remarkably similar to those seen in the intact membrane. The molecular parameters of the receptors indicate an asymmetric shape and a mol. wt in the range of 300,000. It has not yet been possible to obtain forms of lower molecular weight (subunits) which are still capable of binding insulin. The receptors have been purified nearly to homogeneity by the combination of conventional procedures and affinity chromatography on insulin–agarose and wheat germ agglutinin–agarose derivatives. The major problem currently faced in this area is the purification of these molecules in sufficient quantity to perform chemical characterization studies. The paucity of receptors in biological materials can be appreciated by the fact that the isolation of pure insulin receptors using rat liver membranes requires a purification of about 500,000-fold. In contrast, purification of acetylcholine receptors from electric tissues of fish requires a purification of about 100- to 500-fold.

The status of insulin receptors has been examined in several insulin-resistant states. A decrease in insulin binding to liver and fat cell membranes has been observed in the obese-hyperglycemic syndrome of the mouse, a recessively inherited trait characterized by marked obesity and insulin resistance.[8] It is not yet known, however, what this apparent "decrease" means with respect to the physiology of insulin in this abnormal state, and its possible relation to insulin resistance is at present a conjecture. The normal physiological responses to insulin depend on the coordinate behavior of a number of complex biochemical steps, perhaps the simplest and least complex of which is the binding to receptors. Even if consideration of these steps is confined to the cell membrane, it is apparent that the hormone–receptor complex, once formed, must modify in a highly specific and probably very subtle way the activity of other membrane-localized components such as adenylate cyclase, glucose transport permeases, and perhaps also guanylate cyclase and phosphodiesterase. Since the coupling of the initial hormone–receptor interaction to other cellular events is probably quite sensitive to even slight modifications in the membrane composition of phospholipids, carbohydrates, etc., it is perhaps a little too simple to expect that naturally occurring metabolic abnormalities would be likely to originate primarily at the level of the receptor. In addition to the membrane-localized functions which must be finely regulated by the receptor, there are undoubtedly other complex intracellular events which can be modified to produce abnormal responses to the hormone. It is interesting that the number of insulin-binding sites appears to be normal in certain insulin-resistant metabolic states, such as in rats that are starved, treated with prednisone, or made diabetic by administration

of streptozotocin, and in other species which show decreased responsiveness to insulin.[9]

It has recently been demonstrated[10] that exogenous phosphorylation of a specific, low mol. wt protein of fat cell membranes results in the selective suppression of insulin-stimulated (but not basal) rates of glucose transport, but does not modify other insulin responses such as antilipolysis. It will be of interest to determine if this mode of membrane phosphorylation is altered in some of the conditions described above, especially in view of the increasing recognition of protein phosphorylation as an important regulatory mechanism in biology.

Human peripheral white blood cells possess specific binding sites for insulin.[11] However, it has been shown[12,13] that, whereas some leukocytes (e.g. macrophages) can bind considerable amounts of insulin, nylon column-purified lymphocytes free from macrophages, polymorphs and platelets have less than one binding site per cell. Virtually no binding is seen in these cells compared to permanent cell line lymphocytes (RPMI 6237) maintained in tissue culture or to normal lymphocytes transformed *in vitro* by plant lectins.[12] The binding observed to unfractionated human lymphocytes[11] could be explained by a 0·3 per cent contamination with cells such as macrophages or transformed lymphocytes. Consequently, changes in insulin binding measured in such cells (e.g. in disease or in metabolic states of insulin resistance) could reflect small changes in cell composition rather than in insulin receptors.

Recently it has been observed that during mitogenic blast transformation of human lymphocytes by a variety of mitogens, there is a dramatic *de novo* appearance of cell surface receptors for insulin.[12,13] It will be important to determine if such receptors also appear when lymphocytes are subjected to a more physiological stimulus, namely a specific antigen. The emergence of receptors clearly follows activation of RNA and protein synthesis, and it precedes the morphologic changes of transformation. These receptors may thus be related to the continuation of some process occurring after the initial stimulus but before cell division. It is of considerable interest that acute lymphocytic leukemia lymphoblasts (human) have insulin receptors comparable to those of cells transformed by mitogens *in vitro*, whereas cells from human chronic lymphocytic leukemia have a very small (if any) number of receptors.

Although it has been known for some time that insulin can act as a serum-substitute to support the growth of cells in culture, it is recognized that insulin does this only at concentrations far above those which occur *in vivo*. Compared to epidermal growth factor, for example, insulin is indeed a poor mitogen for human fibroblasts.[14] It may be significant that binding studies show that the apparent affinity of insulin for receptors in fibroblasts and lectin-transformed lymphocytes is about an order of magnitude lower than the affinity of receptors in fat cells or liver membranes. Furthermore, the dissociation constant (about $10^{-9}$ M) for insulin in these fibroblasts and lymphocytes is an order of magnitude higher than the physiological concentration of the hormone. The possibility must be entertained that these "receptors" may normally be intended for another, as yet unidentified, serum peptide with growth-promoting and insulin-like properties, and that sufficient cross-reactivity can occur with insulin for them to pose as low-affinity, specific "receptors" for this hormone.[13] Other proteins, such as concanavalin A and wheat germ agglutinin, for example, are potent insulin-like compounds, and they can interact directly with insulin receptors. There is reason to speculate that certain insulin-like peptides in serum

(e.g. somatomedin, nonsuppressible insulin-like activity, multiplication-stimulating activity) may be capable of interacting with insulin receptor structures. Some precedents for overlapping receptor specificity may be found with vasopressin and oxytocin (antidiuresis and myometrial contraction,[15,16] human growth hormone, human placental lactogen and prolactin,[17] and secretin and vasoactive intestinal polypeptide.*

It has recently been shown that the plant lectins, concanavalin A and wheat germ agglutinin, have marked insulin-like activities in isolated fat cells and that these lectins bind directly to insulin receptors.[18,19] Physiological concentrations of insulin can inhibit adenylate cyclase activity[20–23] in isolated liver and fat cell membranes, fat cell ghosts, fibroblasts and isolated membranes of *Neurospora crassa*. This effect is also mimicked by low concentrations of the lectin, concanavalin A. It is possible that the mitogenicity of concanavalin A might be related to its insulin-like actions, and more specifically to its effects on adenylate cyclase activity or a closely related process.[18,19]

Mitogenic stimuli have recently been demonstrated to cause immediate, very large elevations of cyclic GMP in lymphocytes.[24] Insulin has similarly been shown[25] to cause rapid and significant elevations of this cyclic nucleotide in sensitive tissues (fat cells, liver) but not in resting lymphocytes, which lack insulin receptors and which are apparently insensitive to this hormone. Thus, in this activity at least, certain lectins can also be considered to be insulin-like. Recent evidence suggests that there may be important reciprocal relationships between intracellular levels of cyclic AMP and cyclic GMP.[24] Although it is not yet known by what mechanisms these cyclic nucleotides are regulated in a concerted fashion, it is quite possible that such changes may be intimately related to the processes of mitogenesis. The possibility must be considered that mitogenic stimuli are artificially eliciting fundamentally "insulin-like" biochemical responses (e.g. inhibition of adenylate cyclase, elevation of cyclic GMP, suppression of cyclic AMP) which are capable of activating a cell that lacks receptors for insulin or for other normally occurring stimuli which have "insulin-like" activity. In this hypothesis it is anticipated that antigenic activation of the lymphocyte would similarly occur by selectively triggering an "insulin-like" response via highly specific receptors for the antigen on the surface of the lymphocyte.

Under certain circumstances insulin can decrease the intracellular concentrations of cyclic AMP in adipose tissue cells and in liver, and these changes appear to correlate with at least some of the biological effects of insulin in these tissues. Although insulin can decrease the activity of adenylate cyclase in subcellular preparations,[20–23] an effect on cyclic AMP phosphodiesterase[26,27] may also be involved in lowering cellular cyclic AMP. However, certain effects of insulin, like its effect on transport processes, have not yet been adequately explained on the basis of cyclic AMP, and in many situations (in the absence of substances that elevate cyclic AMP levels) the action of insulin cannot be correlated with a gross fall of the intracellular levels of cyclic AMP. The recent demonstration that insulin (and cholinergic drugs) causes a marked rise in the levels of cyclic GMP may eventually help to explain the mechanisms by which insulin modulates metabolic events. The hypothesis has been advanced[25] that a unique insulin–receptor (or cholinergic hormone–receptor) inter-

* B. Desbuquois, personal communication.

action at the cell membrane may act by transforming the substrate specificity of the membrane-localized adenylate cyclase into a form which now expresses preference for GTP rather than ATP. The same membrane-localized enzyme system would be responsible for the synthesis of both nucleotides, and the "balance" of ATP- or GTP-utilizing forms of the enzyme would depend on the relative occupancy of receptors which favor the ATP form (adrenergic hormones, glucagon, etc) compared to the occupancy of those receptors that favor the GTP form (insulin, cholinergic hormones). This could explain the simultaneous "inhibition" of adenylate cyclase activity and elevation of cyclic GMP levels (by enhanced synthesis) through the action of a single, unique hormone–receptor interaction. This hypothesis is attractive because it could explain the effects of insulin (and cholinergic hormones) on a "unitary" basis and it would place both cyclic nucleotides as "initial" chemical mediators. Recently obtained evidence indicates the existence of a membrane-localized guanylate cyclase in isolated membrane preparations whose activity can be stimulated directly by insulin and concanavalin A.*

One of the most exciting and important areas of future research in the field of membrane receptors for hormones is the elucidation of the precise mechanisms by which hormone–receptor complexes, once formed, modify the activity of specific membrane-localized enzymes or transport structures. In the past it has been assumed that the receptors themselves possess specific and separate functions (e.g. ionophores for acetylcholine receptors), or that the receptors are structurally contiguous with other molecules endowed with specific functions (e.g. adenylate cyclase for peptide hormones). It is in fact not necessary to make such assumptions, especially since there may be special advantages to having receptors which in their uncomplexed

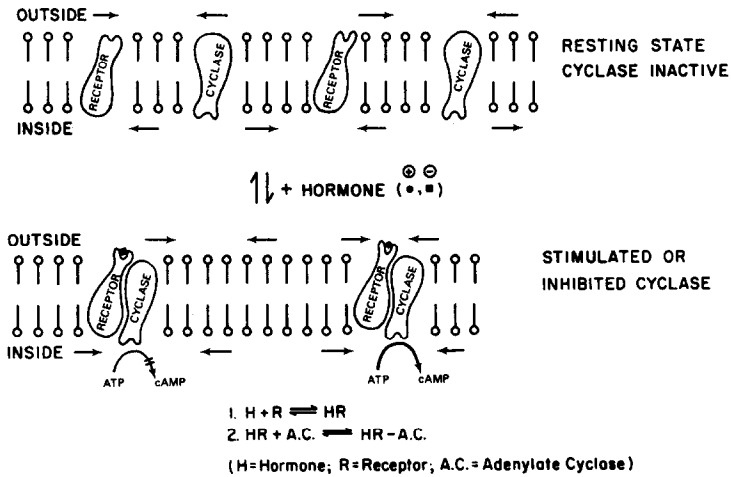


FIG. 1. General two-step fluidity hypothesis for the mechanism of modulation of adenylate cyclase activity of cell membranes by hormones.[1] The central feature is that the receptors and the enzyme are discrete and separate structures which acquire specificity and affinity for complex formation only after the receptor has been occupied by the hormone. These structures can combine after binding of the hormone because of the fluidity of cell membranes. The hormone-binding sites of the receptor are on the external face, exposed to the aqueous medium, and the catalytic site of the enzyme is facing inwardly toward the cytoplasm of the cell.

* M. I. Siegel and P. Cuatrecasas, unpublished.

form are totally separate from other membrane macromolecules. In this theory[1] the receptor assumes new properties upon binding of the specific ligand; one of these new properties is a special affinity for binding to and thus perturbing other membrane structures, such as adenylate cyclase (Fig. 1). This is, then, essentially a two-step mechanism which basically reflects the currently developing view[28] that biological membranes are essentially fluid structures which permit relatively free diffusion of molecules along the plane of the membrane. Sequential specific interactions could therefore occur within the membrane in a fashion analogous to the well known behavior of molecules in aqueous solutions, except that different diffusion properties and special constraints would exist in the former. This two-step membrane hypothesis would help to explain many apparent anomalies in the action of hormones, including how insulin may modify separate and perhaps independent membrane processes by acting on a single receptor and how several hormones acting through independent receptors may modify the same adenylate cyclase in a given cell.[1] In addition, such considerations have important consequences for understanding and predicting the properties and kinetics of the interaction of hormones with membrane receptors.

Insoluble derivatives of peptide hormones, such as insulin–agarose,[29] have been useful in studying the interaction of these hormones with cell surfaces. In properly controlled experiments, where the problem of leakage of ligand from the support is eliminated, the biological activities of some of these derivatives can be demonstrated in isolated cell systems. For instance, insulin–agarose can stimulate fat cells[29] and soluble insulin–dextran macromolecular polymers are active *in vivo* in reducing blood sugar levels and inducing hepatic enzymes.[30–32]

Although the activities of the hormone–polymer derivatives resemble those of the parent active ligand, in some cases qualitative and quantitative differences can be observed. For instance, insulin–agarose appears to be capable of activating fat cells by fewer contacts than the native hormone, and thus in some respects it may be more potent than insulin itself. Similarly, insulin–dextran derivatives appear to be more potent than the native hormone. It is interesting that the peptide, mesenchymal factor, is much more potent in stimulating cell proliferation and cytodifferentiation in rat pancreatic epithelia when attached covalently to agarose than when free in solution.[33] Activity is observed when only 20 derivatized beads are added to the epithelium.

Mammary cells from virgin mice do not respond (α-aminoisobutyric acid transport) to native insulin, but do respond to insulin–agarose, and this activity can be blocked by native insulin.[34,35] Similar kinds of results have been obtained with derivatives of phytohemagglutinin (PHA) and concanavalin A.[36,37] Mouse B-lymphocytes, which ordinarily do not respond (DNA synthesis) well to soluble PHA or concanavalin A, do respond to the agarose–lectin derivatives. Such studies not only emphasize the plasma membrane location of the receptors for these substances and hormones, but they also suggest that the traditionally accepted responses of such receptors are amenable to manipulations by polymeric pharmacologic agents which act outside the cell.

The presence of cell surface receptors may be inferred by direct microscopic observations of cells and agarose beads containing specific hormones. This has been demonstrated with insulin–agarose and fat cells,[38] as well as with leukocytes and

agarose beads containing histamine and norepinephrine.[39] Glass beads containing catecholamines can directly stimulate myocardial cells.[40]

The interaction of insoluble ligand derivatives with cell receptors may differ substantially from that of the soluble ligand. Although this may complicate the interpretation of data on the basis of simple comparisons with monovalent ligands, it also offers new opportunities for exploring special receptor phenomena on cell surfaces. For example, such approaches may be useful in studying the existence and biological consequences of lateral diffusion, migration, aggregation, clustering or other dynamics of receptors within the plane of the membrane.

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