Section II. Mechanism of Action

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The Insulin Receptor

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SUMMARY

Studies with bulky and insoluble insulin derivatives indicate that this hormone can activate various metabolic processes by interaction with cell surface structures. A definition of "receptor" is offered which is operationally useful in studies directed to the identification and purification of receptor structures. Studies are described which measure the specific binding of insulin to intact fat cells and liver and fat cell membranes. The properties of these interactions and the correlation with metabolic events indicate that biologically significant receptors are being studied. The possible contribution of membrane carbohydrates, proteins and lipids to the environment and function of the receptor is considered. Studies pertinent to the extraction and purification of the insulin receptor are discussed. DIABETES *21* (Suppl. 2): 396-402, 1972.

Elucidation of the molecular basis of polypeptide hormone action requires the identification, characterization, isolation and purification of the specific cellular receptor structures with which the hormone initially interacts. For some years there have been theoretical grounds for postulating that the initial and singular locus of insulin action is the cell membrane.[1,2] Only recently, however, has direct experimental evidence been developed to support this view.

Insoluble and bulky derivatives of insulin, prepared by the covalent attachment of the hormone to agarose beads through the α-amino group of the N-terminal residue of the β chain or through the ε-amino group of the lysyl residue, effectively increase the utilization of glucose and suppress hormone-stimulated lipolysis of isolated fat cells.[3] These studies indicate that interaction of insulin with superficial membrane structures alone may suffice to initiate transport as well as other metabolic alterations. These insulin-agarose derivatives are also quite effective in stimulating RNA synthesis[4] and α-aminoisobutyric acid accumulation[5] in isolated mammary cells and in activating glycogen synthetase[6] in liver.

Further support for the effectiveness of this cell surface interaction with insulin was obtained from studies which demonstrated that insulin-agarose (but not unsubstituted agarose) columns retain isolated fat cell ghosts which could be eluted with solutions containing insulin. These studies in addition suggested that these selective absorbents might be very useful for the purification of receptor structures by affinity chromatography.[7] Before such purification procedures could be instituted, however, it was necessary to develop independent procedures for measuring and studying the insulin-receptor interactions of intact cells and cell membranes.

An operational definition of "receptor" has been developed that is meaningful in the recent studies of this laboratory. "Receptor" signifies those molecules of the cell which are uniquely capable of recognizing and interacting with insulin with a high degree of selectivity and affinity and which, in addition, possess the capability of conveying the occurrence of the interaction to biochemical processes resulting in metabolically significant events. Thus, the receptor has at least two functions: (1) to specifically *recognize* insulin and (2) to somehow convey to other molecules the fact that insulin has been recognized. In analogy with all enzyme-substrate systems, these two processes (formation of the precatalytic complex and catalysis) are fundamentally distinct and subject to study independently. It is important that these simple concepts be clearly formulated and understood, since dismantling of the cell to extract and isolate a receptor dissociates the receptor from other structures which are

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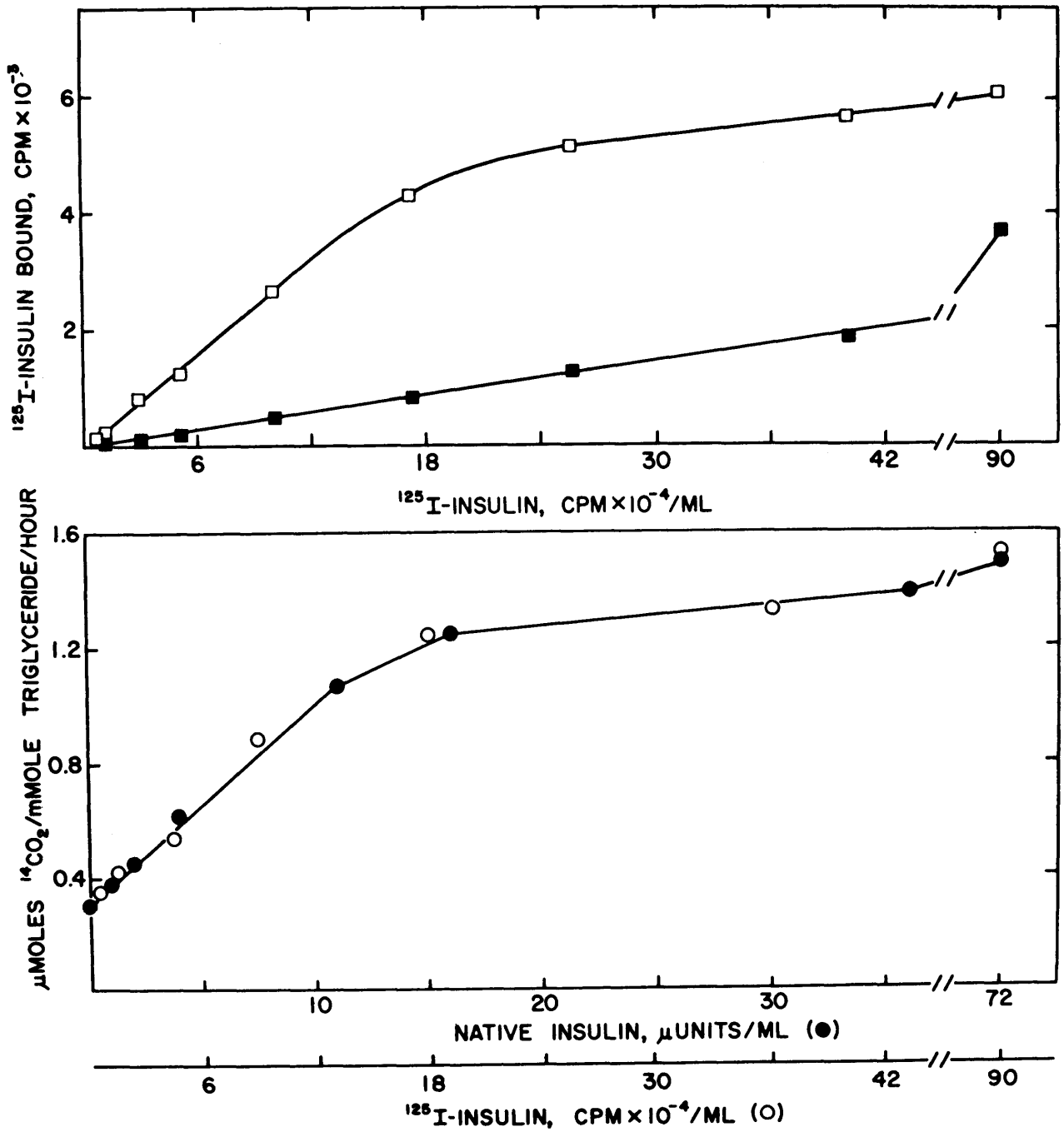

FIG. 1. Ability of native (●) and of ^{125}I-labeled insulin (○) to enhance the rate of glucose oxidation by isolated fat cells (below), correlated with the specific binding of ^{125}I-labeled insulin (□) to fat cells (above). Native insulin assayed 24 U./mg. Fat cells for glucose oxidation were incubated in 1 ml. Krebs-Ringer bicarbonate buffer containing 1 per cent albumin with 0.2 mM [U-^{14}C]glucose (5.1 Ci/mole) for ninety minutes at 37° C. In the binding studies, isolated fat cells (about 2 x 10^5 cells/ml.) were incubated with insulin for twenty minutes at 24° C. in 0.5 ml. Krebs-Ringer bicarbonate buffer containing 1 per cent albumin. For every concentration of ^{125}I-labeled insulin studied, control incubations were performed in the presence of a displacing amount (40 μg.) of native insulin. The nonspecific binding, which is not represented in the curve for specific binding, is plotted in the upper figure (■); this is not a saturable process. (Data from Cuatrecasas.[8])

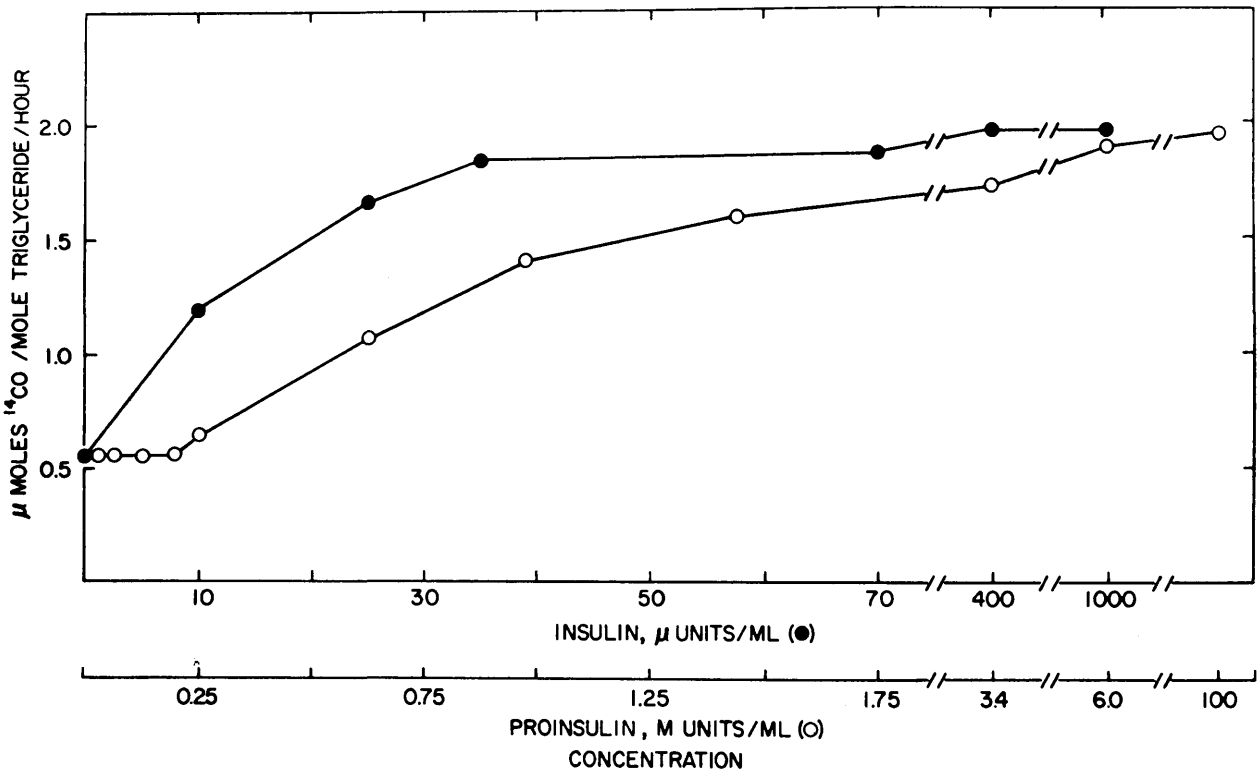

FIG. 2. Effect of increasing porcine insulin (●) and proinsulin (○) concentrations on the conversion of [U-^{14}C] glucose to $^{14}CO_2$ by isolated adipose tissue cells.[3] The cells were incubated for sixty minutes at 37° C. in 1 ml. Krebs-Ringer bicarbonate buffer containing 1 per cent albumin and 0.3 mM [U-^{14}C]glucose. The concentrations are plotted on the basis of 24 U. per mg. of insulin or per 1.5 mg. of proinsulin (M.W. 9,000).

normally necessary to evoke a biologically significant event. The "second" function of the receptor is thus lost. Although a biologically significant system may ultimately be reconstituted from the isolated components of the system, at this stage (cell disruption) identification of the receptor can only be made provisionally by precise measurements and characterization of its recognition function.

For these reasons, the insulin-receptor binding interaction has been studied in great detail in intact fat cells under conditions which permit simultaneous measurement and correlation with the biological response.[8] Once it is established with reasonable certainty that the binding interaction indeed reflects the biologically significant initial interaction, it is possible to disrupt the cell to determine quantitatively the subcellular localization of the insulin binding structure, to compare the properties of the binding interaction with those observed with the intact cell, and to proceed with further extraction and purification with reasonable assurance that the receptor being studied is the same as was originally observed in the intact system. Therefore, the working definition of "receptor" given above is a functional one that is useful experimentally and does not presuppose knowledge of the molecular arrangements of structures vicinal to the receptor. Elucidation of the latter may evolve during study of the receptor.

Insulin binding with fat cell membranes

Procedures have recently been devised which permit direct measurements of binding of ^{125}I-labeled insulin to intact fat cells by methods which are independent of the biological response.[8] Iodinated insulin, as recently reported by Freychet et al.,[9,10] is biologically active. There is excellent correlation between the binding of insulin to cells and its capacity to activate the biological response at similar concentrations (figure 1). Insulin binding is a saturable process. In addition, the binding is time and temperature dependent. Bound insulin is displaced by native insulin and insulin derivatives in proportion to their biological potency. For example, it can be easily demonstrated that the maximal effect of insulin can be achieved with a concentration of proinsulin[11-13] twenty times greater than that of insulin (figure 2). Displace-

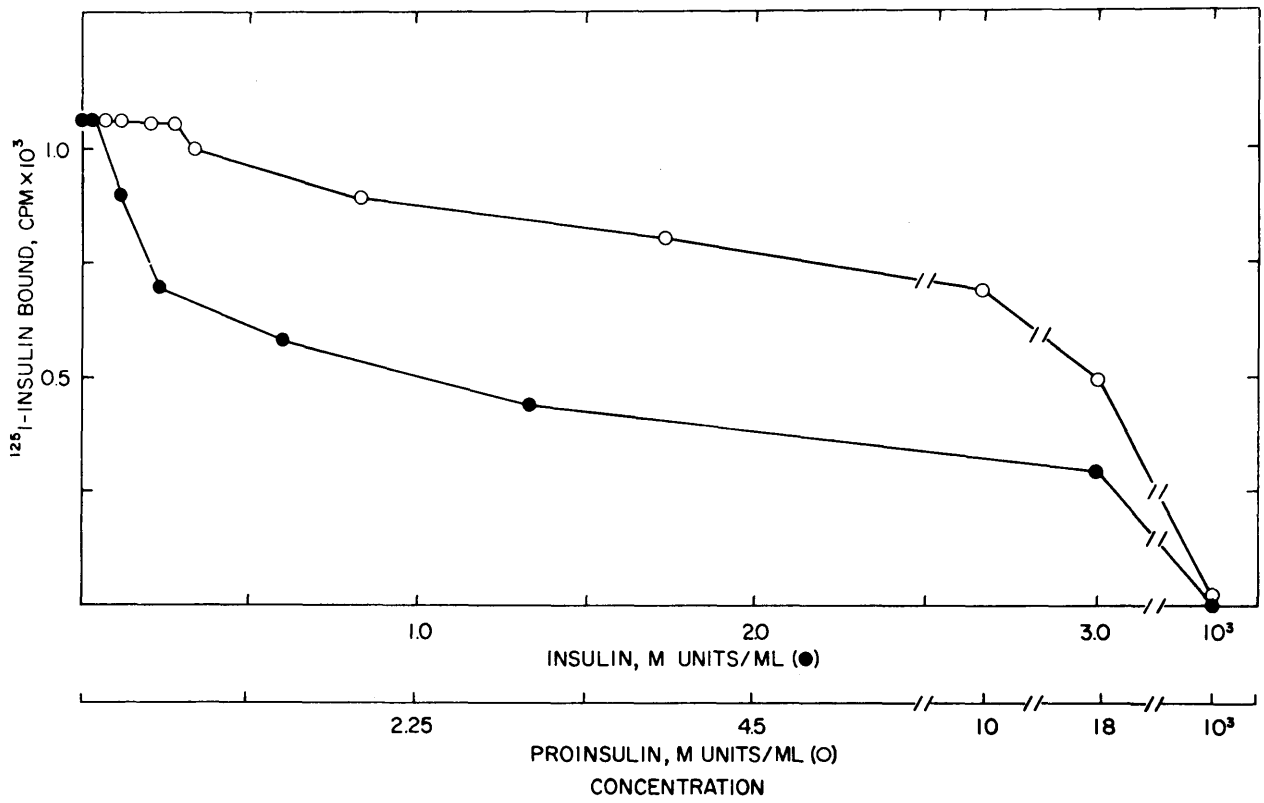

FIG. 3. Effect of native insulin (●) and proinsulin (○) on the displacement of specific ^{125}I-labeled insulin binding to adipose tissue cells. Samples containing about 10^5 cells in 0.2 ml. Krebs-Ringer bicarbonate buffer containing 1 per cent albumin were incubated with 4.4 x 10^{-11} M ^{125}I-labeled insulin for thirty minutes in the presence of the indicated concentrations of insulin and proinsulin. The latter were based on an activity of 24 U. per mg. of insulin and per 1.5 mg. of proinsulin (M.W. 9,000). Specific binding was determined by described procedures.[8]

ment of ^{125}I-labeled insulin by proinsulin similarly requires twenty times greater concentrations than native insulin (figure 3), indicating a twenty fold difference in affinity for the receptor. No displacement of insulin binding is observed with other peptide hormones at very high concentrations (table 1). A maximum of about 11,000 molecules of insulin can bind per cell.

The insulin-receptor association is a bimolecular process with a rate constant of 1.5 x 10^7 M^{-1} $sec.^{-1}$, while the dissociation is a first-order process with a rate constant of 7.4 x 10^{-4} $sec.^{-1}$ On the basis of these rate constants,[8] the dissociation constant of the insulin-cell complex is 5 x 10^{-11} M, which is very similar to the constant obtained from equilibrium data and that calculated on the basis of enhancement of glucose oxidation. This dissociation constant (about 10 μU. of insulin per ml.) is similar to the known concentration of insulin in the serum of fasting individuals.[14] From these data it is reasonable that in this range small variations in concentration would result in alterations of the metabolic state.

TABLE 1

Effect of various polypeptide hormones on the specific binding of ^{125}I-labeled insulin of isolated fat cells.

The indicated hormone was added to 0.5 ml. Krebs-Ringer bicarbonate buffer (1 per cent albumin) containing 2 × 10^5 fat cells. ^{125}I-labeled insulin (2.8 × 10^{-11} M) was added, and after twenty minutes at 24°C. the specific amount of radioactivity bound to the cells was determined.[8]

Hormone	Concentration μg./ml.	^{125}I-labeled insulin bound nanomoles × 10^6
None		2.4
Insulin	0.002	1.4
Insulin	0.012	0.7
Andrenocorticotropin	40	2.4
Growth hormone	40	2.4
Prolactin	40	2.5
Vasopressin	40	2.4
Oxytocin	40	2.4
Glucagon	40	2.5
Carboxymethyl chains of insulin	0.2	2.3
Oxidized chains of insulin	0.2	2.2
Reduced insulin*	0.3	2.2

* Treated for ninety minutes at 24°C. with 20 mM dithiothreitolin 0.1 M $NaCHO_3$ buffer, pH 8.1.

Formation of the insulin-cell complex does not result in chemical changes or inactivation of insulin or of the receptor.[8] Thus it would appear that the action of insulin is not coupled to degradation of the hormone and that no stable covalent forces or disulfide interchange (between insulin and receptor) reactions are involved in the interaction of insulin with its receptor. Furthermore, under conditions necessary to achieve binding equilibrium, no significant inactivation of the insulin occurs in the medium.

It is possible to recover quantitatively in the particulate fraction of a homogenate of fat cells the total binding capacity of the intact cells.[8] No specific binding of insulin can be detected to the mitochondrial or nuclear fractions. Furthermore, the soluble cellular fraction does not effectively compete with the binding of insulin to membranes.

The above studies indicate that the insulin-cell receptor interaction is a simple dissociable process involving a kinetically homogeneous species which is probably present exclusively in the cell membrane. Direct evidence for the *exclusive* localization of insulin receptors to the surface of the cells has been obtained by measuring the specific binding of insulin to membranes from homogenates of fat cells previously digested with trypsin-agarose.[15,16]

An important implication of these studies, as well as those to be described shortly, is that, until compelling reasons to the contrary are forthcoming, it must be assumed that all of the metabolic consequences of insulin should be explicable on the basis of interaction with a single, unique class of receptors. Thus, the view that a unitary or fundamental mediator (chemical or conformational) exists which modulates all of the biological effects of the hormone gains credibility from the present series of studies.

The technics described in this report have been used to explore the possibility that defects in the insulin receptor might explain certain metabolic states characterized by insulin resistance. It has already been demonstrated in rats that conditions such as hydrocortisone administration, streptozotocin-induced diabetes and starvation, which cause a profoundly diminished insulin response in fat cells, are not associated with quantitative or qualitative alterations in the insulin receptors of these cells.[15] Similarly, fat cells from certain species, which in vitro demonstrate very poor insulin effects on glucose transport, do not have significantly diminished receptors or altered affinity for insulin.[17]

As noted above, fat cell membranes contain all of the insulin-binding capacity detectable in the intact fat cell. Furthermore, the detailed kinetic properties of the insulin-fat cell membrane interaction are nearly identical to those observed in the intact cell, suggesting that cell breakage does not adversely affect the binding function of the receptor.[18] It is also of interest that the specific binding of ^{125}I-labeled insulin to purified liver membranes[9,10,18,19] has many properties which are almost identical to those of fat cell membranes (table 2), suggesting that the receptor structures may be similar or identical in these two tissues.[18,19] This is important since in the liver it has not been possible to study the interaction of insulin with intact cells to compare correlation of binding with biological activity. By analogy with the fat cell membranes, however, the liver membrane binding structures are almost certainly the biologically important receptor structures. This is especially important in studies of receptor purification because the scarcity of material from fat tissue is a very serious drawback. This can be partially circumvented by using liver membranes, since they are obtainable in large quantity.

Chemical structure of insulin receptors

The possible contribution of membrane carbohydrate, protein and lipid structures to the integrity and function

TABLE 2

Equilibrium and kinetic constants for the specific interaction of insulin with isolated fat cells, fat cell membranes, and liver cell membranes

Constant	Membranes		
	Fat[a]	Liver[b]	Fat Cells[c]
Association rate (k_1, mole^{-1} sec^{-1})	8.5×10^6	3.5×10^6	1.5×10^7
Dissociation rate (k_{-1}, sec^{-1})	4.2×10^{-4}	2.7×10^{-4}	7.4×10^{-4}
Dissociation constant			
from k_1/k_{-1}	5×10^{-11}M	7.7×10^{-11}M	5×10^{-11}M
from equilibrium data	7.5×10^{-11}M	6.7×10^{-11}M	8×10^{-11}M

[a]Data from Cuatrecasas.[18]
[b]Data from Cuatrecasas et al.[19]
[c]Data from Cuatrecasas.[8]

of the insulin receptor has been studied by measuring the biological and receptor effects of digesting intact cells and membranes with neuraminidases,[20] proteolytic enzymes[21] and phospholipases and lipid-active agents.[22] These studies suggest that the insulin receptor may be glycoprotein having surface sialopeptides which are not essential for binding of insulin but which may be important in the second function (transmitting a signal) of the receptor. These terminal structures are probably highly exposed to the solvent and are very susceptible to cleavage by free or agarose-bound enzymes in the aqueous medium. A variety of proteolytic enzymes seem to hydrolyze these terminal glycopeptides at more proximal regions than are modified by carbohydrases. Although such modification does not destroy the receptor binding function, it causes a severe fall in the affinity of the receptor for insulin. Removal of membrane phospholipids exposes more critical regions of the receptor, since under such conditions the insulin-binding function of the receptor is totally destroyed by protease concentrations too low to cause any significant effects on the native cell or membranes.

One of the more interesting but least understood observations is the unmasking of new insulin-binding structures upon removal of membrane phospholipids from fat or liver membranes.[18,22] Such manipulations can result in a sixfold increase in the total insulin-binding capacity of the membranes. These receptors are not normally accessible to macromolecules (proteases, insulin) in the aqueous medium, but they are readily modified by compounds of low molecular weight, such as tetranitromethane. It appears that these receptors are not functionally coupled to the biological effects of insulin, at least not in isolated fat cells studied in vitro. Whether this "reservoir" of receptors can be overtly expressed, thus acquiring functional expression under certain in vivo states, is not known. Possibly such structures are merely a vestige of an earlier developmental stage in which greater quantities of insulin receptors are required.

As indicated earlier, chemical derivatives of insulin bind to the receptor in proportion to their biological effectiveness. Further support for the identification of the binding function as a receptor function is found in the various studies of cell surface modification. It has not been possible in any of the studies performed so far to cause alterations of the binding of insulin to fat cells without at the same time causing a quantitatively similar dysfunction in the metabolic effects of insulin in these cells.[20-22] The only means of dissociating these two processes is by selective destruction of the metabolic processes or by uncoupling them. Furthermore, selective alterations of the receptor appear to cause alterations in the response of glucose transport to insulin which are very similar to the alterations in the antilipolytic effects of insulin. There are, of course, means of destroying the biological response to insulin (e.g. neuraminidase digestion) without affecting the insulin-binding function. Although not yet demonstrated, it may somehow be possible to selectively alter one of the biological effects of insulin without affecting other metabolic effects by interfering with processes occurring distal to the insulin-receptor interaction. By themselves, such effects would neither suggest the existence of multiple classes of receptor nor indicate that the fundamental chemical event in insulin action is different for the various metabolic processes. Until convincing evidence to the contrary is forthcoming, it must be assumed that a single and homogeneous class of insulin receptor exists, and that the interaction of these receptors with insulin leads to a unique "biochemical event" which ultimately finds expression in varied portions of the cell in perhaps equally varied metabolic parameters. Other regulatory influences, and the sensitivity of these various metabolic processes to the basic "biochemical event," would dictate the precise total biological response to insulin under any given circumstances.

It is of interest that the binding function of the insulin receptor is not grossly dependent on membrane phospholipids, since delipidation does not impair this function.[22] Very recently the insulin receptor has been extracted quantitatively from liver and fat cell membranes in water-soluble form.[23] Early characterization of this isolated receptor suggests that it is an asymmetric protein with a molecular weight of about 300,000. It does not appear to be a lipoprotein.[15]

Insoluble derivatives of insulin prepared by coupling the hormone to agarose or glass beads are quite effective in extracting from membrane suspensions those particles which contain specific insulin receptors. It is hoped that these derivatives will be useful in the purification of the solubilized insulin receptor.

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