

Insulin-Receptor Interactions in Adipose Tissue Cells: Direct Measurement and Properties

(association constant/cell membrane/glucagon/HTC cells/erythrocytes)

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ABSTRACT An assay system is described for measurement of specific binding of [125 I]insulin to intact fat cells and membrane fractions from such cells. The binding is time- and temperature-dependent and saturable with respect to insulin; the bound insulin is displaced by native insulin but not by oxidized or reduced insulin or by a number of other peptide hormones. A maximum of about 11,000 molecules of insulin can bind per cell. The insulin-receptor association is a bimolecular reaction with a rate constant of $1.5 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$, while the dissociation is a strictly first-order process with a rate constant of $7.4 \times 10^{-4} \text{ sec}^{-1}$. A dissociation constant of $5.0 \times 10^{-11} \text{ M}$ can be calculated from these rate constants, whereas a value of $6.1 \times 10^{-11} \text{ M}$ is obtained on the basis of enhancement of glucose oxidation. Complex formation does not result in chemical change or inactivation of insulin or receptor. The total binding capacity of fat cells is quantitatively recovered in the particulate fraction after homogenization. The insulin-cell receptor interaction is a simple dissociable process involving a homogeneous species probably present exclusively in the cell membrane.

The proposition (1) that many, and perhaps all, of the diverse metabolic effects of insulin result from interactions of this hormone with the cell membrane has been experimentally substantiated by demonstrating that insulin-agarose derivatives incapable of penetrating isolated adipose tissue cells are biologically active (2). Studies of the properties of the postulated insulin receptor have been based on subjecting fat cells to proteolytic (3) and chemical (4) procedures which presumably perturb cell-surface structures. Unfortunately, all such studies have been based on measurements of very complex metabolic parameters (e.g., glucose oxidation, antilipolysis) that are distant or terminal consequences of the primary insulin-receptor interaction. This report describes the utilization of simple and sensitive procedures for *direct* measurement and study of specific insulin-receptor interactions.

METHODS AND PROCEDURES

Insulin is iodinated at 24°C by addition of 4.0 μg of crystalline porcine zinc-insulin, in 10 μl of 0.1 M sodium phosphate buffer, pH 7.4, to 100 μl of 0.25 M sodium phosphate buffer, pH 7.4, containing 1.0 mCi of Na^{125}I . 20 μl of chloramine-T (5) (5 mg/ml of water) are added and, after 40 sec, 20 μl of sodium metabisulfite (10 mg/ml of water). 8 ml of 0.1 M

sodium phosphate-0.1% albumin buffer is added immediately. The radioactivity precipitable by trichloroacetic acid is determined. One talc tablet (25 mg) is added and dispersed in the solution. The talc pellet obtained by centrifugation (10 min at 2000 rpm) is resuspended and centrifuged four times with 0.1 M sodium phosphate buffer, pH 7.4. The [125 I]insulin is eluted from the talc by addition of 3 ml of 0.37 N HCl containing 6% (w/v) bovine albumin. The suspension is centrifuged for 30 min at 2500 rpm, and the supernatant is neutralized by addition of 0.5 ml of 1 N NaOH. Recovery of iodoinsulin adsorbed to talc is 80-90%. The [125 I]insulin obtained has 0.7 atom of ^{125}I per mole of insulin, and 95% of the radioactivity is precipitated with 20% trichloroacetic acid, 97% is adsorbed to talc, and 98% is adsorbed to micro-fine silica, QUSO G-32, Philadelphia Quartz Co. (6); the conditions are described in the footnotes to Table 2. The specific activity of [125 I]insulin is 1.45 Ci/ μmol . Radioactivity is determined by liquid scintillation using 10 ml of TLX toluene fluoralloy and 2 ml of Bio-Solv Solubilizer BBS-3 (Beckman). Counting efficiency, with an iodine-125 gamma ray standard, is 55%.

The standard binding assay consists of incubating fat cells ($0.5-5 \times 10^5$ cells) at 24°C for 20 min in 0.5 ml of KRB-1% albumin containing [125 I]insulin. At 24°C equilibrium is achieved within 20 min under virtually all conditions tested. 3 ml of ice-cold KRB-0.1% albumin is added to the cells, which are immediately filtered and washed with another 10 ml, under reduced pressure, on cellulose acetate EAWP Millipore filters. The steps of dilution, filtration, and washing consume about 15 sec. The filters are removed, cut in halves, and placed in counting vials containing 1 ml of 10% (w/v) sodium dodecyl sulfate; after shaking for 30-40 min at 24°C, 10 ml of the counting solution is added. All samples are done in triplicate or quadruplicate. The choice of membrane for filtration is a critical factor since insulin adsorbs strongly to most cellulose derivatives.

"Specific" binding is obtained by subtracting from the total radioactive uptake the amount that is not displaced by high concentrations (40-80 $\mu\text{g}/\text{ml}$) of native insulin. This nonspecific component is related linearly to the concentration of iodoinsulin (Fig. 1, *upper*), and is independent of time or temperature of incubation. It represents nonspecific adsorption to filter (80-90%) and to the cells, and it constitutes about 0.1-0.3% of the total radioactivity passed through the filter. This proportion is much (and prohibitively) higher in

Abbreviations: KRB-1% albumin, Krebs-Ringer bicarbonate buffer containing 1% (w/v) bovine albumin; HTC cells, hepatoma tissue culture cells.

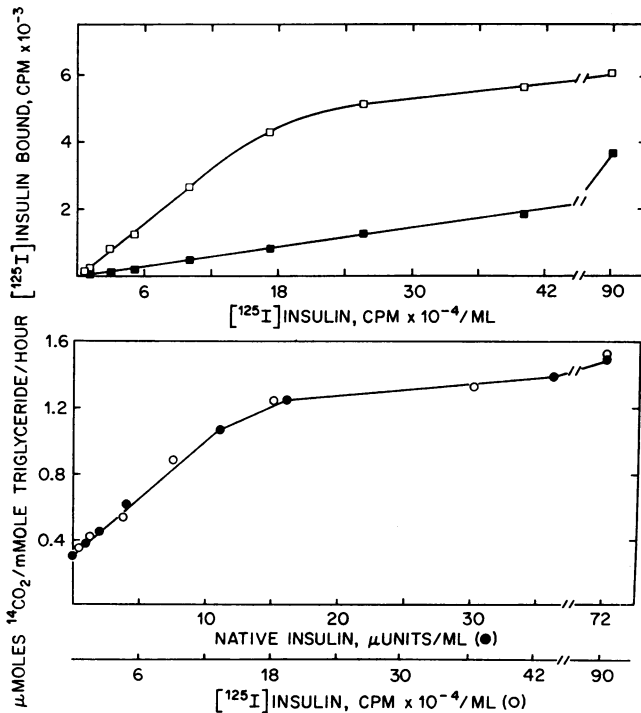


FIG. 1. Ability of native (●) and of [¹²⁵I]insulin (○) to enhance the rate of glucose oxidation by isolated fat cells (below), correlated with the specific binding of [¹²⁵I]insulin (□) to fat cells (above). Native insulin had 24 units/mg. Oxidation was in 1.0 ml of KRB-1% albumin, 0.2 mM [¹⁴C]glucose (5.1 Ci/mol) for 90 min at 37°C. In the binding studies, isolated fat cells (about 2 × 10⁶ cells/ml) were incubated with insulin for 20 min at 24°C in 0.5 ml of KRB-1% albumin. For every concentration of [¹²⁵I]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.

improperly purified or otherwise damaged preparations of [¹²⁵I]insulin.

Isolated fat cells were prepared by the method of Rodbell (7), and the conversion of [U-¹⁴C]glucose to ¹⁴CO₂ was determined as previously described (2). Hepatoma tissue culture (HTC) cells were provided by Drs. B. Thompson and J. Miller, and erythrocyte ghosts by Dr. V. Marchesi, National Institutes of Health.

RESULTS

Biological activity of [¹²⁵I]insulin

The ability of [¹²⁵I]insulin to enhance the conversion of [¹⁴C]glucose to ¹⁴CO₂ by isolated fat cells is indistinguishable from that of native insulin over the entire range of insulin concentration (Fig. 1, lower). These results are in accord with earlier reports which indicate that incorporation of less than 1 atom of iodine per mole of insulin does not lead to biological inactivation (8).

Binding as a function of [¹²⁵I]insulin concentration

The specific binding of [¹²⁵I]insulin to isolated fat cells is a saturable process with respect to [¹²⁵I]insulin concentration (Fig. 1, upper). There is excellent correlation between this

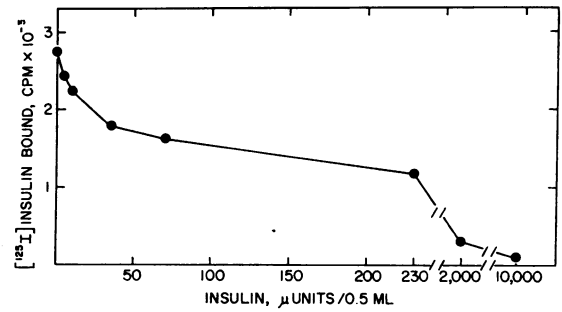


FIG. 2. Displacement by native insulin (24 units/mg) of [¹²⁵I]insulin specifically bound to isolated fat cells. Fat cells, in 0.2 ml of KRB-1% albumin, were incubated at 24°C for 30 min with 1.9 × 10⁻¹¹ M [¹²⁵I]insulin and increasing amounts of native insulin. The nonspecific binding (400 cpm) has been subtracted from all experimental points.

binding curve and the dose-response curve of glucose oxidation (Fig. 1, lower). Thus, if intact metabolically responsive cells are used in the assay, it is possible to ascertain with reasonable validity that the biologically active receptor is being studied.

Displacement of bound [¹²⁵I]insulin by native insulin

[¹²⁵I]Insulin specifically bound to fat cells is displaced by increasing concentrations of native insulin (Fig. 2) in a fashion predicted by the near identity of these two molecules. In contrast (see below), several other peptide hormones cause no displacement even at high concentrations.

Binding as a function of cell concentration

The binding of [¹²⁵I]insulin is directly proportional to the cell (or receptor) concentration over the range of cell concentrations that can be used in these procedures (Fig 3). This linear relationship in the absence of saturating concentrations of insulin is a reflection of the high affinity of the interacting species. It can be compared to the early, linear portion of the curve of Fig. 1, upper. It is difficult to achieve sufficiently high concentrations of cells to clearly demonstrate a plateau of binding. However, such a plateau is observed in binding

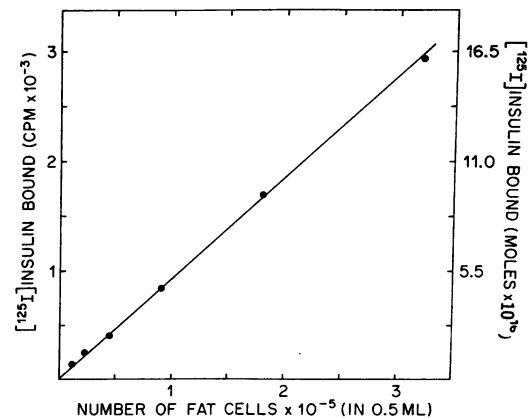


FIG. 3. Specific binding of [¹²⁵I]insulin to isolated fat cells as a function of the concentration of fat cells in the medium. The fat cells from 10 rats were suspended in 7 ml of KRB-1% albumin and serial dilutions were made with the same buffer. The cells, in a total volume of 0.5 ml, were incubated for 30 min at 24°C in 1.4 × 10⁻¹⁰ M [¹²⁵I]insulin.

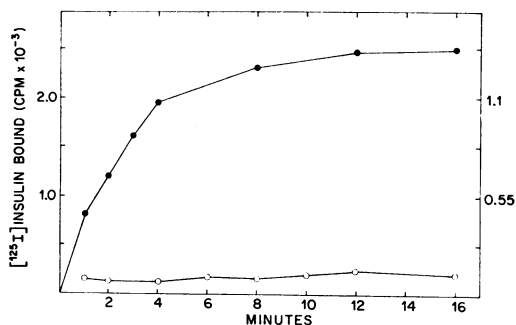


FIG. 4. Rate of binding of [^{125}I]insulin to isolated fat cells at 24°C . Fat cells ($3 \times 10^6/\text{ml}$) were incubated with 6.8×10^{-11} M [^{125}I]insulin in the presence (O) and absence (●) of native insulin ($40 \mu\text{g}/\text{ml}$). The left ordinate describes the uptake of radioactivity; the right, the corresponding concentration of the complex that was used to calculate the kinetic constants.

studies that utilize isolated fat-cell plasma membranes, which can be tested in higher concentrations (unpublished).

Rate constants of insulin-receptor interaction

The specific binding of [^{125}I]insulin to fat cells is a time-dependent process (Fig. 4). In contrast, the binding that is not displaced by native insulin is at its maximum in the shortest incubation period. At 4°C the rate of specific binding is decreased at least 10-fold, but the nonspecific binding is again at its maximum after incubation for less than 1 min.

It is possible to utilize the rate data (Fig. 4) to calculate the bimolecular rate constant of insulin-receptor association, k_1 . Since 10^6 cells can bind a maximum of 1.8×10^{-14} mol of insulin (Fig. 1), one cell can bind 11,000 molecules of insulin. The latter therefore can be used to determine directly the concentration of receptor, assuming that the biologically active species of insulin is the monomer (2). The strictly bimolecular nature of the association process is established by

TABLE 1. Ability of adipose tissue cells to bind [^{125}I]insulin after dissociation of cell-insulin complex with insulin antiserum

Suspensions of fat cells (5.0×10^6 cells in 2 ml) were incubated for 20 min at 24°C in KRB-1% albumin in the absence ("no prior treatment") and presence ("prior treatment") of 2.9×10^{-10} M [^{125}I]insulin. $10 \mu\text{l}$ of undiluted guinea pig anti-porcine insulin serum was added to some of the suspensions, and the incubation was continued for 40 min. The cells were washed twice with 12 ml and resuspended in 7 ml of the same buffer. Cell samples (0.5 ml) were then incubated for 20 min at 24°C in the presence of 1.4×10^{-10} M [^{125}I]insulin to determine specific binding.

| Cell treatment | Specific binding of [^{125}I]insulin (fmol/ 10^6 cells) |
|--|--|
| No prior treatment, no antiserum | 1.21 ± 0.03 |
| No prior treatment, + antiserum | 1.15 ± 0.04 |
| Prior treatment, no antiserum | 1.24 ± 0.02 |
| Prior treatment, + antiserum | 1.17 ± 0.03 |
| Prior treatment, + antiserum, not washed | 0.18 ± 0.01 |

Values are means \pm SE ($n = 3$). fmol = femtomoles (10^{-15} mol).

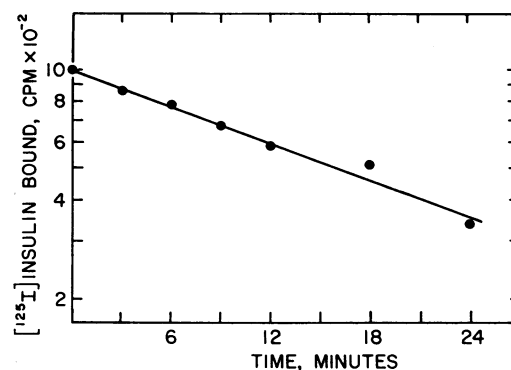


FIG. 5. Semilog plot of the dissociation of [^{125}I]insulin bound to fat cells as a function of time at 24°C . Adipose cells ($2 \times 10^6/\text{ml}$) were incubated for 20 min in 0.5 ml of KRB-1% albumin containing 4.6×10^{-11} M [^{125}I]insulin. The cell suspension was rapidly diluted with 3 ml of ice-cold buffer containing 0.1% albumin, filtered under reduced pressure on Millipore membranes, and washed with 10 ml of the same buffer; these steps consumed about 15 sec. Suction was discontinued and 15 ml of KRB-1% albumin, at 24°C , was added on top of the filters. The filtration rate was then about 0.5 ml/min. At intervals the buffer was rapidly removed by vacuum filtration, and the radioactive content of the filters was determined.

the fact that constant values of k_1 are obtained when the experimental points along the curve in Fig. 4 are substituted into the second-order equation, $k_1 = 2.303/t(a-b)[\log b(a-x)/a(b-x)]$, where a is $[I]$, b is $[R]$, and x is the amount of a reacting in time t . At 24°C the value for k_1 is $1.5 (\pm 0.4) \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$. This rate probably approaches that of a diffusion-controlled process, particularly in view of the extraordinary size of one of the diffusing species.

The rate of dissociation (k_{-1}) of the insulin-receptor complex, which can be measured directly, is strictly a first-order process (Fig. 5). This rate constant at 24°C is $7.4 (\pm 0.3) \times 10^{-4} \text{ sec}^{-1}$. The half-life of the complex is 16 min.

Properties of insulin and receptor previously involved in complex

Virtually complete dissociation of the cell-insulin complex can be brought about with a large excess of insulin antiserum, and the ability of cells to bind [^{125}I]insulin again after such dissociation is not impaired (Table 1). There is no evidence that a ternary cell-insulin-antibody complex can occur.

The [^{125}I]insulin tightly complexed to the cells can be eluted and shown to behave exactly like freshly prepared [^{125}I]insulin in terms of some physical properties that sensitively detect alterations of the insulin molecule (Table 2). The eluted insulin can bind again with cells (Table 2), and with insulin antibody, at least as well as the untreated [^{125}I]insulin. Furthermore, no alteration in physical properties or binding ability is detected in the [^{125}I]insulin of a filtrate obtained after incubation of cells with 2×10^{-11} M insulin for 1 hr at 24°C . The [^{125}I]insulin eluted from intact fat cells is not present in sufficiently high concentrations to permit adequate tests of biological activity. However, [^{125}I]insulin eluted from fat-cell membrane fragments is unaltered in its capacity to enhance glucose oxidation of fat cells (unpublished). There is no evidence that at physiologic concentrations there is any alteration of insulin during the binding process or otherwise by exposure to fat cells.

TABLE 2. Characterization of [¹²⁵I]insulin eluted from insulin-fat cell complex

Isolated fat cells from 8 rats were suspended in 10 ml of KRB-1% albumin and treated with phospholipase C* (20 μg/ml) for 40 min at 37°C. The cells were washed twice with 20 ml of the same buffer, suspended in a volume of 10 ml, and incubated for 20 min at 24°C with 1.3×10^{-10} M [¹²⁵I]insulin. 1-ml samples were diluted with 3.5 ml of cold KRB-1% albumin, filtered on EAWP Millipore filters, and washed with 10 ml of the cold buffer. The filters were cut into small pieces and pooled. The [¹²⁵I]insulin was eluted by shaking the filters in 3 ml of 0.2 N HCl-10% albumin (w/v) for 30 min at 24°C. The eluted material was neutralized with 1 N NaOH. The recovery of [¹²⁵I]insulin, based on the residual radioactivity present in the filters and that eluted, was 75%.

| Properties | [¹²⁵ I]Insulin | |
|--|----------------------------------|-------------------|
| | Not used previously | Eluted from cells |
| % Precipitable by 20% TCA† | 95 | 97 |
| % Adsorption to talc† | 85 | 95 |
| % Adsorption to QUSO G-32‡ | 94 | 95 |
| Specific binding to cells | <i>fmol/10⁶ cells</i> | |
| 4.8×10^{-12} M [¹²⁵ I]insulin | 0.30 | 0.39 |
| 2.4×10^{-12} M [¹²⁵ I]insulin | 0.12 | 0.16 |

* The enhancement of insulin binding after digestion of cells with phospholipase C will be described separately in detail (manuscript in preparation).

† Buffer: 0.1 M sodium phosphate (pH 7.4)-0.6% (w/v) albumin. TCA, trichloroacetic acid.

‡ Buffer: 0.1 M sodium acetate (pH 5.1)-0.6% (w/v) albumin. QUSO G-32, microfine silica (Philadelphia Quartz Co.).

Effects of various peptide hormones on insulin binding

Evidence for the specificity of the insulin-cell interaction is further strengthened by the failure of several polypeptide hormones to compete with insulin for binding to cells. We observed no displacement of [¹²⁵I]insulin (2.8×10^{-11} M) binding to cells (4×10^5 per ml) by adrenocorticotropin, growth hormone, prolactin, vasopressin, oxytocin, or glucagon at concentrations of 40 μg/ml. The lack of binding of oxytocin is especially noteworthy in view of the similarity of ring structure and overlap in biological activity of oxytocin and insulin; it has been postulated that they can at least partially share their cellular receptors (reviewed in ref. 9).

The S-sulfonated or carboxymethyl insulin chains, singly or in combination, do not displace [¹²⁵I]insulin at a concentration of 0.2 mg/ml. Likewise, insulin (0.3 mg/ml) reduced by 90-min incubation in 50 mM dithiothreitol (0.1 M NaHCO₃ buffer, pH 8.1) does not compete with [¹²⁵I]insulin. Albumin does not modify the binding at concentrations as high as 5% (w/v), although a 45% decrease in binding is observed with 10% albumin.

Effect of cell fractionation

Homogenization of fat cells beyond the point that intact cells are recognizable does not alter the total amount of insulin receptor (Table 3). The receptor is quantitatively recovered in the particulate, nonnuclear fraction. The effect of digesting this particulate material with various enzymes is identical with that of the intact cells (unpublished). Membrane

TABLE 3. Effect of homogenization and centrifugation of fat cells on the specific binding of [¹²⁵I]insulin

Isolated fat cells (3×10^6 cells/ml), suspended in 8 ml of KRB-1% albumin, were homogenized with a Brinkmann Polytron Pt-10 (2.2 setting). The pellets were suspended in the same buffer. Phospholipase C* (40 μg/ml) digestion was at 37°C for 40 min. Assay with 1.8×10^{-10} M [¹²⁵I]insulin.

| | Total [¹²⁵ I]insulin-binding capacity (fmol) |
|--|--|
| Initial cell suspension | 12.3 |
| Ditto, treated with phospholipase C | 34.1 |
| Homogenization, 30 sec | 11.4 |
| Homogenization, 90 sec | 12.8 |
| Supernatant ($23,000 \times g$ for 30 min) | 2.1 |
| Pellet ($23,000 \times g$ for 30 min) | 13.2 |
| Pellet treated with phospholipase C | 37.4 |
| $750 \times g$ pellet from $23,000 \times g$ pellet† | 0.9 |

* P. Cuatrecasas, manuscript in preparation.

† Numerous free nuclei were found by phase-contrast microscopy.

"ghosts" prepared from fat cells by osmotic shock (10) also possess intact receptors.

DISCUSSION

The "binding" of insulin to various tissues has been extensively studied in the past (for review see ref. 2). These studies, however, do not discriminate among "binding" of insulin to intracellular, membrane, or connective tissue structures, and the results have been inconclusive and contradictory. Although it has been demonstrated that the primary interaction of insulin is with the cell membrane (2), it has not been possible to study the interaction of insulin with the presumed membrane receptor by procedures that are independent of complicated biological functions. Furthermore, it is not known whether insulin can, in addition to its membrane interaction, penetrate the cell interior in significant quantity.

The procedures described in this report permit direct, sensitive, and quantitative observations of complex formation between insulin and cell receptors at concentrations of hormone normally present in biological fluids. The interaction is a highly specific, temperature-sensitive, dissociable process endowed with high affinity. It is possible to measure saturation with respect to insulin, and to determine separately the rate constants for association and dissociation. The specific kinetic data obey simple rate equations, a reflection of the uniformity and homogeneity of the receptor and the absence of irreversible chemical alterations of the interacting species. The measurements are made on isolated, metabolically active cells, and there is a close parallel between binding and biological activation. These data indicate that a biologically significant and unique interaction is indeed being studied, and that only insignificant quantities of insulin can gain access to other cellular compartments.

The dissociation constant of $I + R \rightleftharpoons IR$, on the basis of the k_{-1}/k_1 ratio, is 5.0×10^{-11} M. This compares favorably with the value of 8.1×10^{-11} M calculated on the basis of the concentrations of free I and R, and of IR, at equilibrium under nonsaturating conditions. The concentration of native

TABLE 4. Specific binding of [¹²⁵I]insulin and [¹²⁵I]glucagon to fat cells, HTC cells, and erythrocyte ghosts

| | Concn (M) | | Binding (fmol/10 ⁶ cells) | |
|--------------------|-----------------------|-----------------------|--------------------------------------|--------------------|
| | Glucagon | Insulin | Glucagon | Insulin |
| Fat cells | 1.5×10^{-10} | 1.3×10^{-10} | 0.21 | 0.37 |
| HTC cells | 8.6×10^{-11} | 9.7×10^{-11} | 0.01* | 0.024 |
| Erythrocyte ghosts | 2.5×10^{-11} | 2.9×10^{-11} | 8×10^{-6} | 5×10^{-6} |

Cells were incubated for 20 min at 24°C in KRB-1% albumin.

* Not significant.

insulin required for half-maximum stimulation of glucose oxidation (Fig. 1) is 6.1×10^{-11} M. These values are near those in the serum of normal human subjects, small variations of which result in overall metabolic alterations (11).

The insulin-receptor interaction does not result in inactivation or significant chemical alteration of either interacting component, and complex formation does not involve formation of stable covalent bonds. Thus, the receptor cannot complex with insulin through a sulfhydryl-disulfide interchange reaction as has been suggested (reviewed in ref. 9), and cell activation is not coupled to insulin degradation.

The insulin-binding capacity of fat cells is quantitatively recovered in the particulate fraction after homogenization. The effects of chemical and enzymatic perturbations, and the kinetics of insulin binding, are nearly identical in the intact cell and in the membrane fraction (unpublished). These considerations strengthen the contention that no significant receptors occur outside the plasma membrane. Furthermore, detailed studies can now be performed on isolated membrane preparations with some assurance that these represent biologically significant observations.

On the basis of the techniques and observations described here, it has been possible to study directly the consequences of modifying the insulin structure and the surface of the cell with chemical and enzymatic probes (unpublished). In addition to

permitting direct studies on the possible mechanism of insulin action on adipose tissue cells, these procedures can be applied also to the study of the interaction of other peptide hormones on these and other cells. For example, we find that [¹²⁵I]-glucagon can bind specifically to fat cells but not to HTC cells (Table 4). Insulin binds quite well to the latter cells, but binding to erythrocyte ghosts is about 10⁵ times less effective than to fat cells.

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