INSULIN-INDUCED DISSOCIATION OF ITS RECEPTOR INTO SUBUNITS: POSSIBLE MOLECULAR CONCOMITANT OF NEGATIVE COOPERATIVITY

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SUMMARY

The detergent-solubilized avian insulin receptor retains negative cooperativity and other binding properties of the membrane bound form. On gel filtration the receptor elutes as a single peak with a Stokes radius of 72 Å. Preincubation of the receptor with low levels of insulin leads to the formation of a second, smaller form with a Stokes radius of 40 Å. The percent of receptor in this second peak is proportional to the insulin concentration and correlates well with the insulin-induced increase in dissociation rate (negative cooperativity). Both the isolated high molecular weight and the isolated low-molecular-weight forms of the receptor re-equilibrate in the presence of insulin and, upon refiltration of either isolated peak, both forms of the receptor are obtained. These results are compatible with a model of the insulin receptor in which a tetrameric form can dissociate to a monomeric form as a concomitant of negative cooperativity.

The binding of insulin to its receptor at the cell surface is not a simple bimolecular reaction. Equilibrium studies have revealed curvilinear Scatchard plots, and kinetic studies have shown an insulin-induced acceleration of the rate of dissociation of receptor-insulin complexes. On the basis of these and other findings, we have proposed that insulin receptors exhibit site-site interactions of the negative cooperative type and that the receptor can exist in high and low affinity states (1-3). The conversion of the receptor from the high affinity to the low affinity state can be produced by occupancy of the receptor binding sites by insulin as well as by low pH, high temperature, urea and anti-receptor antibodies (3,4).

A number of models for negative cooperativity of surface receptors have been proposed, involving interactions among subunits (3,5,6) or movement of proteins within a "fluid mosaic" membrane (7-9). We have previously demonstrated that the solubilized insulin receptor still exhibits site-site interactions indicating that a planar membrane is unnecessary (10). To further characterize the molecular events occurring during these cooperative interactions we have measured the size of the receptor by gel filtration in the presence and absence of insulin. In this study we find that insulin induces a reversible change in the Stokes radius of the receptor from 72 to 40 Å and that the percentage of receptors in the smaller form correlates well with the insulin-induced increase in dissociation rate.

METHODS

^{125}I-insulin was prepared as previously described (3) and further purified by gel chromatography over Sephadex G-50. Avian erythrocyte membranes were purified (11), dissolved in 1% Triton X-100, centrifuged at 100,000 X g and dialyzed into 0.1% Triton. Receptor binding activity was determined by adding 50 µl of the fraction to be assayed to a mixture of ^{125}I-insulin (17 pM) in 85 mM Tris-HCl, 30 mM NaCl, 10 mM glucose, 0.05% Triton X-100, 1 mg/ml bovine serum albumin, pH 7.3 to give a total volume of 0.5 ml. The mixture was incubated for 4 hours at 15°. The bound hormone was separated by precipitation with 12.5% polyethylene glycol (12) and collected on cellulose acetate filters. The ^{125}I-insulin bound in the presence of 2 µg/ml insulin was considered to be non-specific binding.

Gel filtration of the receptor was performed on Sepharose 6B. The detergent solubilized receptor was incubated for 4 hours at 15° with buffer or buffer containing unlabeled insulin (25 or 50 ng/ml). The incubation mixture was then chilled to 4°, applied to a Sepharose column that had previously been equilibrated with the same insulin concentration, and filtered at 4°. Each column was calibrated with Blue Dextran 2000 and NaCl to determine the excluded and included elution volumes and each run contained a ^{125}I-thyroglobulin marker (12 S). The fractions from each column were diluted 1:10 and assayed for insulin binding activity. Inhibition of the assay by the diluted insulin still present in the elution buffer was determined by mixing experiments using fractions before the void volume and was 40 and 60% for buffers containing 25 and 50 ng/ml of insulin. Concentrations above 50 ng/ml, even at 1:10 dilution, caused sufficient inhibition in the assay to preclude their use. In experiments to test reversibility of the size change, the receptor was incubated with 50 ng/ml of insulin, gel filtered, assayed and the regions of desired activity were pooled. The pooled fractions were concentrated ten-fold using a Schleicher and Schull protein concentrator with nominal MW cut-off of 25,000 and dialyzed against 50 ng/ml insulin for 3 days. Each fraction was then incubated for 4 hours at 15° and gel filtered over Sepharose 6B which had been equilibrated with 50 ng/ml insulin. Data are expressed as counts/min (10 minute counting period) of ^{125}I-insulin specifically bound. The recovery of insulin binding activity from the Sepharose columns ranged from 90 to 105%.

RESULTS

Solubilized insulin receptors, from avian erythrocytes membranes, freshly prepared or allowed to incubate for 4 hours at 15° in the absence of insulin, eluted as a single peak of binding activity slightly after the 12 S thyroglobulin marker (Figure 1, top panel). This position was indicative of a Stokes radius of 72 Å and was consistent with a molecular weight of 300,000 (13,14). In contrast, incubation of the receptor with 25 ng/ml of insulin and filtration of the mixture over a Sepharose 6B previously equilibrated with 25 ng/ml insulin yielded two peaks of insulin-binding activity (Figure 1, lower panel). In addition to the major peak, which eluted at the position of native receptor, a smaller, more included, peak was observed. This smaller peak accounted for 17.5 ± 1.5 percent of the insulin binding activity (mean ± standard error of the mean for three experiments) and eluted at a volume indicative of a Stokes radius of 40 Å, consistent with a molecular weight of 75,000. When the same experiment was performed with 50 ng/ml of insulin the percentage of the

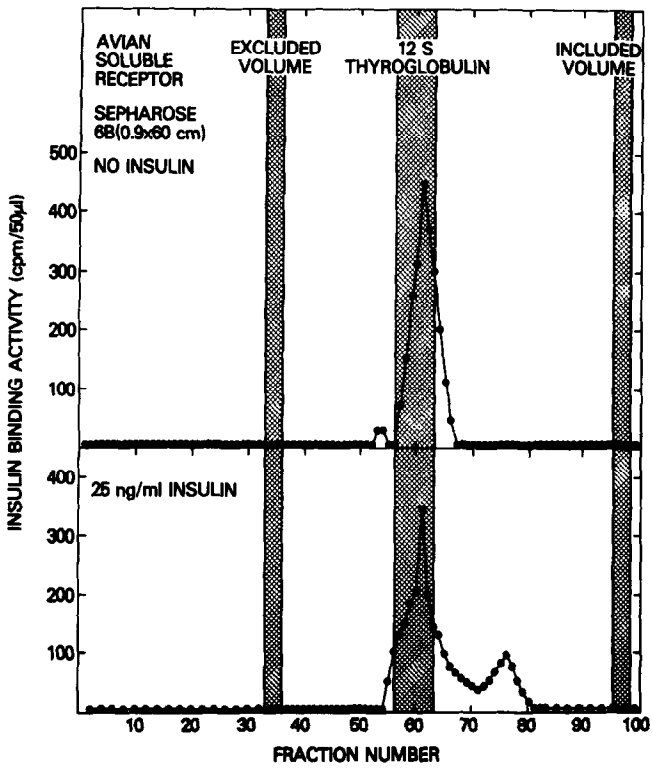

FIGURE 1: Gel filtration of the insulin receptor. Upper Panel: Detergent-solubilized receptor was incubated with buffer for 4 hours at 15° and then run over a Sepharose 6B column (0.9 X 60 cm) along with a 12 S ^{125}I-thryoglobulin marker. Each fraction was assayed as described in Methods and the results expressed as specific cpm insulin binding activity of the fraction. Lower Panel: Experimental details were as in Upper Panel except 25 ng/ml of insulin were included in the incubation and elution buffers and the column had also been previously equilibrated with this buffer.

small, more included, peak increased to 29 ± 2.5 percent (Figure 2). The 72 Å and 40 Å receptor peaks showed similar concentration dependence for competition by unlabeled insulin of ^{125}I-insulin binding (data not shown). This was probably due to re-equilibration of the forms (see below).

The percentage of insulin-binding activity in the more included fractions was dependent only on the amount of insulin in the incubation buffer. Varying the concentration of Triton X-100 (0.05-0.2%) or addition of other proteins, such as bovine serum albumin, to the incubation and elution buffers did not alter the distribution of binding activity. Furthermore, low pH, which induces a decrease in receptor affinity, did not alter receptor size. Thus, insulin receptor which was incubated and gel filtered at pH 6.0 showed a profile of binding activity identical to that seen in Figure 1, upper panel.

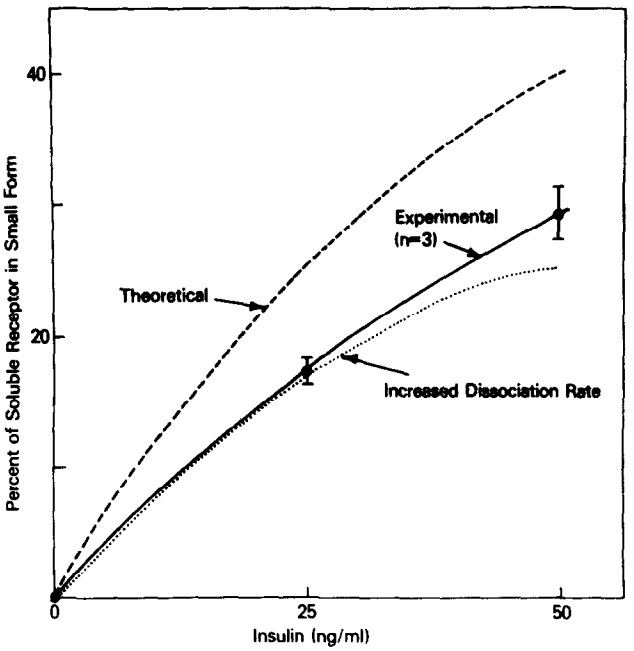

FIGURE 2: Effect of insulin concentration on gel filtration of insulin receptor. Experimental details were identical with Figure 1. The experiment was repeated three times each at 0, 25, and 50 ng/ml of insulin and the amount of binding activity in the smaller, more included peak is expressed as a percent of the total recovered binding activity (90-105%) of that added to the column). The dashed, theoretical line is derived from the model of De Meyts (2) and the dotted, increased dissociation line from experiments with the soluble receptor (10).

The change in Stokes radius of the insulin receptor was reversible. When the receptor was incubated and filtered in the presence of 50 ng/ml insulin (Figure 3, panel A) and the fractions comprising each of the two peaks (I & II) were pooled, concentrated, and refiltered in Sepharose 6B in the presence of 50 ng/ml of insulin, each of the pooled fractions gave rise to both peaks of insulin binding activity. Refiltration of the large forms of the receptor (peak I) yielded both the large and small forms with the small form accounting for about 34% of the total binding activity. Similarly refiltration of the small form of the receptor, peak II, yielded both forms of the receptor; again, the second peak accounted for 33% of the total binding activity. Thus, each form of the receptor was capable of generating both forms, if kept in the presence of insulin.

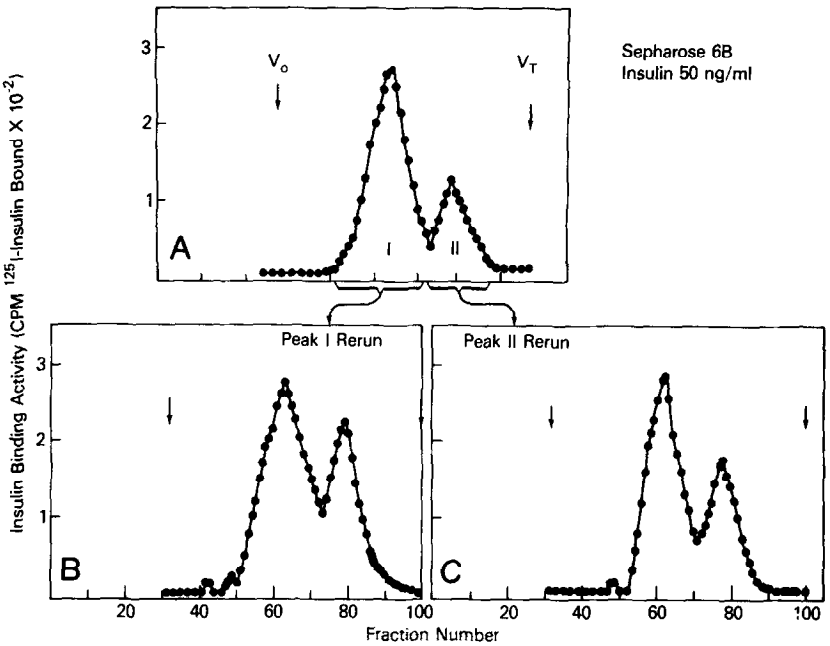

FIGURE 3: Reversibility of receptor size change. A. Experimental details identical with Figure 1, lower panel, except the buffers contained 50 ng/ml of insulin and the column size was larger (2.5 X 100 cm). The regions indicated by the brackets were pooled, concentrated and rerun. B and C. Peak I and II, respectively, rerun on Sepharose 6B (0.9 X 60 cm) in the presence of 50 ng/ml of insulin.

DISCUSSION

In the absence of insulin, the soluble insulin receptor of the avian erythrocyte appears as a single peak on gel filtration with a Stokes radius of 72 Å. In the presence of insulin a second peak of insulin binding activity with a Stokes radius of 40 Å is seen. The affinity of each peak for insulin was the same and on refiltration each isolated peak yielded both forms of the receptor. The initial incubation of 4 hours at 15° is sufficient for insulin to cause a substantial shift of native to small receptor, yet during the gel filtration, which required 6-8 hours at 4°, the forms elute as separate peaks. Thus, the kinetics of interconversion of the forms must be slow at 4°.

The change in receptor size is specific for insulin, and the quantity of the second, more included peak, is directly related to the insulin concentration. There is also a close correlation between the amount of receptor in the small form and the insulin-induced increase in the dissociation rate (Figure 2, dotted line). Thus, insulin at 25 ng/ml induced a 17.5% shift

in receptors and a 17% increase in the dissociation rate, while these values for 50 ng/ml were 29 and 25%, respectively (10). At pH 6 the affinity of the receptor is lowered, but only the native form of the receptor is seen on gel filtration.

Hormone-induced alterations in receptor size have previously been described in two other systems. Glucagon induces a decrease in molecular weight of the myocardial glucagon receptor of at least 4-fold (15). Conversely, the gonadotropin releasing hormone receptor on pituitary cells, which demonstrates positive cooperativity of hormone binding, increases in size upon ligand binding (16).

The change in Stokes radius of the insulin receptor may be due to a change in the shape of the receptor, a change in its partial specific volume or a change of the number of interacting subunits. However, a change in Stokes radius from 72 to 40 Å would necessitate a change of an oblate ellipsoid with an axial ratio of 25 to a sphere or an increase in density of about 5-fold. A more reasonable explanation is the dissociation of the receptor into 4 subunits

In a recent thermodynamic model of the insulin receptor, we have demonstrated that for a cooperative system in which the receptor exists in low and high affinity states, the minimum number of interacting sites is a function of the ratio of the two affinity constants, if one assumes the shift is quantal (2). The ratio of the high to low affinity binding constants for the soluble avian erythrocyte is 10 and, for this value, the model predicts at least 4 interacting subunits. This same number would also explain a shift in Stokes radius of 72 to 40 Å. Using this model it is possible to calculate theoretically the amount of receptor in the two affinity states at any insulin concentration. A plot of the percent receptor in the low affinity state (Figure 2, dashed line) is only slightly higher than the curve of experimental values of the percentage receptors in the small form.

Taken together these findings suggest a model for the insulin receptor such as that shown in Figure 4. In this model, interaction of insulin with any of the 4 sites of the tetrameric high affinity conformation (squares) would cause a small conformation shift in all of the subunits (circles) and would result in a decreased affinity of all of the subunits for insulin (i.e., negative cooperativity). This change might also result in a decreased affinity of the receptor subunits for each other thereby causing reversible size change. This change in Stokes radius is a concomitant of negative cooperativity, but is probably not a prerequisite for the affinity change for several reasons: (a) at 4° negative cooperativity is rapidly induced by insulin, yet at this temperature the receptor size change appears to be slow; (b) lowering the pH to 6.0 induces the low affinity state, yet only the 72 Å form

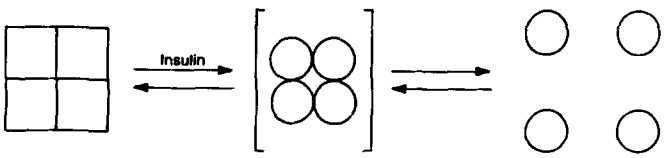

FIGURE 4: Model of dissociating subunits of the insulin receptor.

of the receptor is observed on gel filtration; and (c) the deviation from the theoretical curve shown in Figure 2 suggests that the percentage of receptors in the low affinity state is greater than the precent of receptors of the smaller size. Thus a model of a high affinity tetramer which in the presence of insulin or lowered pH shifts to a low affinity tetramer and in the presence of insulin dissociates to a low affinity monomer would explain our gel filtration and binding data and is compatible with the thermodynamic model.

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