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THE INSULIN-RECEPTOR INTERACTION: IS THE KINETIC APPROACH FOR INFERRING NEGATIVE-COOPERATIVE SITE-SITE INTERACTIONS VALID?

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The dissociation of insulin from its receptor is reportedly enhanced when the dissociation is induced by dilution in the presence of insulin. This experiment is frequently conducted when curvilinear Scatchard plots of insulin binding are observed in order to infer negative cooperative site-site interactions amongst insulin receptors. However, when insulin binding to purified liver plasma membranes was measured at 15°C in 50 mM Tris, pH 7.5 containing 0.1% bovine serum albumin and 100 U/ml bacitracin, the insulin binding data was characterised by a linear Scatchard plot and a Hill plot with a slope equal to unity. Thus, under the conditions of this binding assay, insulin apparently bound to a single non-interacting class of homogeneous binding sites. But, despite the apparent absence of cooperative interactions under these specific conditions, the dissociation of receptor-bound insulin was still enhanced when the dissociation of insulin from its receptor was induced by dilution in the presence of insulin. This result cast serious doubt on the validity of inferring negative-cooperative site-site interactions amongst insulin receptors based solely on the observation that the dissociation of receptor-bound insulin is enhanced by dilution in the presence of insulin.

The Scatchard plot is invariably used for the initial analysis of thermodynamic equilibrium binding data obtained from studies of reversible ligand-receptor interactions (1). In this analysis, the ratio of the concentrations of the receptor-bound ligand to the free ligand is plotted on the abscissa against the concentration of the free ligand on the ordinate. Where the data conform to a straight line, the equilibrium constant for the ligand-receptor interaction is derived from the slope of

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the line and the receptor concentration is derived from the intercept of the line at the ordinate. However, many ligand-receptor interactions are complex and are characterised by curvilinear Scatchard plots. These curvilinear Scatchard plots are most frequently interpreted as being due to the presence of a heterogeneous population of binding sites or to negative-cooperative site-site interactions amongst receptors. According to the now classical experiment first described by DeMeyts *et al.* (2), negative-cooperative interactions can be invoked to explain the observed curvilinearity of the Scatchard plots if the dissociation rate of the receptor-bound ligand is enhanced when the dissociation is induced by dilution in the presence of the ligand. But there has been a continuing debate over the interpretation of this kinetic type of experiment and its validity for implicating negative cooperative interactions (reviewed in reference 3).

Numerous reports have appeared describing curvilinear Scatchard plots of insulin binding to its receptor. The general concensus of opinion has been that the binding is of the negative-cooperative type since most studies have confirmed the earlier work of DeMeyts *et al.* that the dissociation rate of receptor-bound insulin was enhanced when the dissociation was induced by dilution in the presence of insulin (2, 4). However, several recent studies have reported linear Scatchard plots of insulin binding under certain stringent conditions (5-11). Indeed, in the first studies on the properties of the insulin receptor, Cuatrecasas reported a linear Scatchard plot of insulin binding to liver plasma membranes (12, 13). When I measured insulin binding to liver plasma membranes at 15°C in 50 mM Tris, pH 7.5 containing 0.1% bovine serum albumin and 100 U/ml bacitracin, I also found that the binding was characterised by a linear Scatchard plot. Yet curiously, under the same conditions, the rate of dissociation of receptor-bound insulin was still enhanced when the dissociation was induced in the presence of insulin. The implications of this finding are discussed.

EXPERIMENTAL PROCEDURES

Materials- Bovine serum albumin and bacitracin were purchased from Sigma Chemical Co. (St. Louis, MO). [[^{125}I] Tyr^{A14}]Iodoinsulin was purchased from Amersham (Australia). Single peak porcine insulin was purchased from the Commonwealth Serum Laboratories (Melbourne, Victoria). All other chemicals were of reagent grade and obtained from commercial sources.

Preparation of Liver Plasma Membranes- Plasma membranes were prepared from the livers of 175-200 g male Wistar rats according to the method of Ray (14). The 5`-nucleotidase activity of these membranes was concentrated about 30 fold in comparison to the crude liver homogenates.

Insulin Binding to Plasma Membranes- Plasma membranes (12.5 μg membrane protein) were incubated in 0.2 ml of 50 mM Tris, pH 7.5 containing 0.1% bovine serum albumin, 100 U/ml bacitracin, 8 fmoles [[^{125}I]Tyr^{A14}]iodoinsulin (50-75 TBq/mmole) and unlabelled insulin over a pM to μM range of concentration. Insulin bound to the plasma membranes was separated from the unbound insulin by collecting the membranes on Whatman GF/C glass fibre filters (2 cm diameter) under vacuum. These filters were washed with 10 ml of ice-cold 50 mM Tris, pH 7.5 containing 0.1% bovine serum albumin immediately before collection of the plasma membranes and with 2 ml of the same buffer after collection of the membranes. The filters were removed and their radioactivity determined.

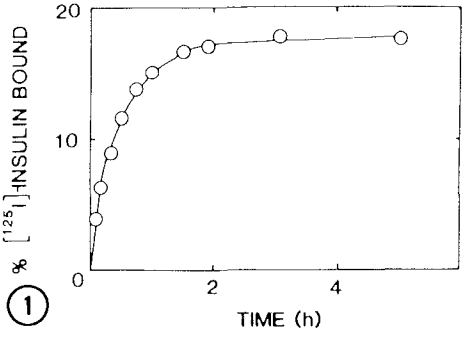
Dissociation of Receptor-Bound Insulin- Plasma membranes (450 μg membrane protein) were incubated in 7.2 ml of 50 mM Tris, pH 7.5 containing 0.1% bovine serum albumin, 100 U/ml bacitracin and 8 fmoles [[^{125}I]Tyr^{A14}]-iodoinsulin (50-75 TBq/mmole). The plasma membranes were then pelleted by centrifugation (10,000 x g for 10 min at 15°C) and the supernatant containing the free insulin was discarded. The plasma membrane pellet (about 450 μl) was rapidly resuspended at 15°C in 180 ml of 50 mM Tris, pH 7.5 containing 0.1% bovine serum albumin, 100 U/ml bacitracin in the presence or absence of 25 nM native insulin. Whatman GF/C glass fibre filters were washed with 10 ml 50 mM Tris, pH 7.5 containing 0.1% bovine serum albumin and then 5 ml aliquots of the diluted plasma membranes were collected by filtration on the filters under vacuum at various times after dilution. The filters were finally washed with 2 ml of ice cold 50 mM Tris, pH 7.5 containing 0.1% bovine serum albumin before removing them to determine their radioactivity.

Analysis of Binding Data- Binding data were analysed by the method of Scatchard (1) using the `LIGAND` program of Munson and Rodbard (15). The data were fitted with either a one-site model or a two-site model. The goodness of fit of the model was analysed with the `LIGAND` program using the `Runs test` of Bennett and Franklin (16). This test predicts whether or not the scatter of points about a fit is due

to chance and therefore, whether the model chosen provides a significant fit to the data. Whether or not the more complex two-site model provided a statistically better fit over the one-site model was tested with the `LIGAND` program using an F-test criterion on the residual variances of the two models. Non-specific binding represented less than 0.5% of the total [^{125}I]-iodoinsulin bound and was handled as a computer fitted parameter. All points within an analysis were weighted equally. The binding data were also analysed using the Hill plot (17). Estimates of B_m (the maximum amount of ligand that can be bound) and non-specific binding were derived from the Scatchard plots of the binding data.

RESULTS

Insulin binding to highly purified liver plasma membranes was monitored as a function of time to establish the incubation conditions required to reach equilibrium (Fig. 1). A steady state equilibrium was reached after incubation for 4 hours at 15°C. This steady-state equilibrium was maintained for at least an additional 2 hours. More than 98% of the total insulin (receptor-bound and free) was precipitable in 5%



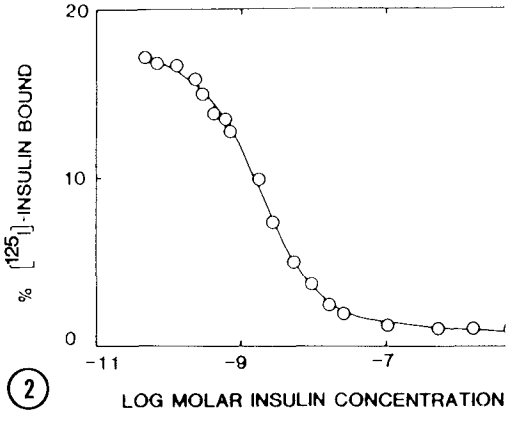
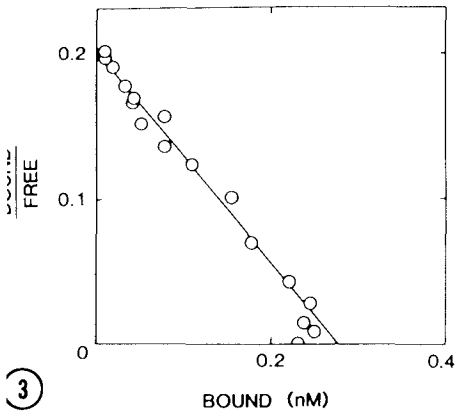


Fig. 1. Time course of insulin binding: the percentage of [^{125}I]-iodoinsulin bound to liver plasma membranes was measured as a function of time according to the experimental procedures.

Fig. 2. The binding of [^{125}I]-iodoinsulin as a function of the total insulin concentration: the percentage of [^{125}I]-iodoinsulin bound to liver plasma membranes was measured according to the experimental procedures. The total insulin concentration was calculated as the sum of the native insulin and the [^{125}I]-iodoinsulin. The curve connecting the experimental points (o) represents the best fit of a one-site model to the data using the `LIGAND` program as described in the experimental procedures. Each point represents the mean of five replicates.



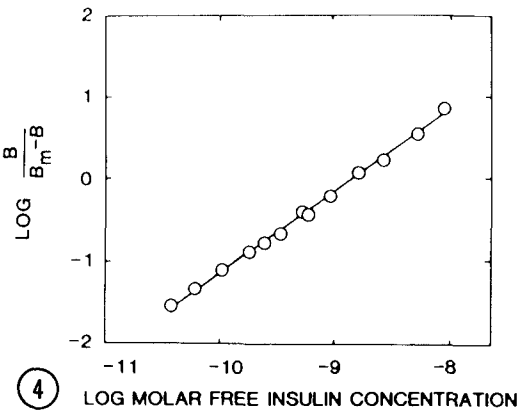


Fig. 3. Scatchard plot of insulin binding: data from Fig. 2 were analysed by the method of Scatchard (1) using the `LIGAND` program as described in the experimental procedures. The line connecting the experimental points (o) represents the best fit of a one-site model to the data.

Fig. 4. Hill plot of insulin binding: data from Fig. 2 were analysed using the Hill plot as described in the experimental procedures. A linear regression was fitted to the data. B and Bm represent the concentration of bound insulin and the maximal concentration of bound insulin respectively.

trichloroacetic acid after a 4 hour incubation at 15°C. The time taken to reach equilibrium was not dependent on the insulin concentration.

The binding of [^{125}I]-iodoinsulin to liver plasma membranes was measured in the presence of a range of insulin concentrations (Fig. 2). The Scatchard and Hill plots derived from these data are illustrated in Fig. 3 and Fig. 4 respectively. Different models were fitted to the data using the `LIGAND` program as described in the experimental procedures. A one-site model provided a good fit to the Scatchard plot. More complex models could not be fitted to the data. The equilibrium constant and the total receptor concentration derived from the Scatchard plot fitted with the one-site model were estimated to be 1.4 $\pm$ 0.1 nM and 4.4 $\pm$ 0.1 pmoles/mg of plasma membrane protein respectively. The Hill coefficient was calculated to be 1.0 from the slope of the Hill plot which had a linear correlation coefficient of 0.999.

The rate of dissociation of insulin from its receptor was followed by measuring the percentage of receptor-bound insulin remaining with

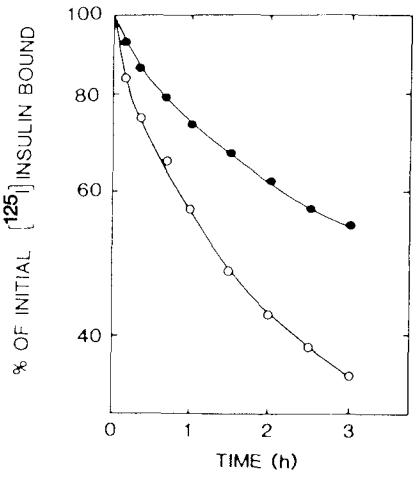


Fig. 5. Dissociation of receptor-bound insulin: the dissociation of receptor-bound [^{125}I]-iodoinsulin was induced by dilution in the presence (o) or absence (●) of 25 nM native insulin as described in the experimental procedures.

time after a rapid 400-fold dilution (Fig. 5). The rate of dissociation of the receptor-bound insulin was accelerated in the presence of buffer containing 25 nM insulin. The dissociation profiles did not follow simple first-order kinetics whether insulin was present or absent from the dissociating buffer. However, the profiles were well fitted by a model assuming a rapidly (approx. 10^{-3} s^{-1}) and a slowly (approx. 10^{-5} s^{-1}) dissociating component.

DISCUSSION

Several assumptions are implicit in the analysis of binding data using Scatchard or Hill plots. Firstly, it is assumed that binding is measured at a true thermodynamic equilibrium. In this study, a steady state equilibrium was acheived that was stable for several hours. This equilibrium was not perturbed by insulin degradation which was negligible when bacitracin was included in the incubation buffer. Thus, I beleive that the binding data was being analysed at, or at least close to, the true thermodynamic equilibrium. Secondly, it is assumed that the binding equilibrium is not perturbed during the separation of the bound and free ligand. In this study, the bound insulin was separated from the

free insulin by collecting the plasma membranes onto glass fibre filters. If this procedure perturbed the separation of the bound and free insulin, it would be expected that the estimate of bound insulin would be dependent on the volume of the buffer used to wash the plasma membranes following their collection onto the filters. I found that the estimate of bound insulin was independent of the volume of the washing buffer from 2 to 25 ml (data not shown). Hence this assumption appears to be valid. Thirdly, it is assumed that the interaction between the ligand and the receptor is reversible. Fig. 5 illustrates that more than 60% of the bound ^{125}I-iodoinsulin was dissociated from the plasma membranes following just 3 hours dilution in the presence of 25nM unlabelled free insulin and the dissociation increased to 98% of the bound insulin at 24 hours after dilution (data not shown). Thus, the insulin-receptor interaction was reversible. Fourthly, it is assumed that the labelled and unlabelled ligands behave identically. In this study, insulin monoiodinated exclusively on its A^{14}-tyrosine residue was utilised since this insulin derivative has been shown to have a biological potency indistinguishable from native insulin (18). Thus, I am confident that the assumptions made in analysing the insulin binding data in this study are all valid.

The insulin binding in this study was characterised by a linear Scatchard plot (Fig. 3) and a Hill plot (Fig. 4) with a Hill coefficient equal to unity. These results imply that under the conditions of the binding assay, insulin was binding to a single non-interacting class of homogeneous binding sites. But despite the apparent lack of cooperative interactions under these conditions, the dissociation of receptor-bound [^{125}I]-iodoinsulin was accelerated when native insulin was present during the dilution-induced dissociation of the receptor-bound insulin (Fig. 5). Since the interpretation of negative cooperativity is always linked with curvilinear Scatchard plots, the present result therefore casts considerable doubt on the value of depending on the enhanced

dissociation of receptor-bound insulin in the presence of native insulin to validate negative-cooperative site-site interactions amongst insulin receptors. Others have also presented arguments against the kinetic approach for inferring negative-cooperative interactions amongst insulin receptors but the validity of many of these arguments has been disputed (3). Moreover, some other ligand-receptor systems show linear Scatchard plots and an enhanced dissociation when the dissociation is induced in the presence of the ligand (19,20). It is also interesting to note that accelerated dissociation of bound [^{125}I]-iodoinsulin by native insulin has been demonstrated for biologically inert substances such as talc (21).

Although I cannot exclude the possibility that negative-cooperative site-site interactions occur amongst insulin receptors, the findings of this study provide direct evidence that the kinetic approach used to infer negative-cooperative site-site interactions amongst insulin receptors is invalid.

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