KINETICS OF ^{125}I-INSULIN BINDING TO RAT LIVER PLASMA MEMBRANES

P.D.R. HOUSE
Department of Biochemistry, School of General Studies, Australian National University, Canberra, A.C.T., Australia

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1. Introduction

There is increasing evidence [1–5] which suggests that insulin interacts with a plasma membrane 'receptor site' (or sites) in a number of whole and fragmented tissues. Many questions connected with the physical properties of insulin binding to plasma membranes from various sources still remain to be answered. In a previous report [6] we showed that a single particulate fraction of rat liver plasma membranes was capable of binding ^{125}I-insulin. The amount bound greatly exceeded the binding to other fractions of plasma membranes isolated from the same liver. Further work on this particulate membrane fraction indicates that the early phase of insulin binding has a complex time course.

2. Methods

Rat liver plasma membranes were prepared as described previously [6], with the following modification. The plasma membranes isolated at the 35%–0.25 M sucrose interface on a discontinuous gradient [7], following centrifugation at 75,500 g_{ave} for 90 min, were found to correspond to the particulate insulin-binding fraction (PL_3) previosuly isolated on a Ficoll gradient [6]. This fraction was used throughout the present work, and greatly diminished the time required for preparation.

Insulin was iodinated by the ICI method [8] to a specific activity of 2 μCi/μg. An aggregated form of iodinated insulin was isolated on Sephadex G-50 (fine) using 40 mM sodium phosphate buffer (pH 7.4), 0.1% bovine serum albumin and 0.6 mM thiomersalate as the eluant. ^{125}I-insulin that did not show an aggregation pattern on elution from Sephadex did not bind to the plasma membranes [7]. The biological activity of the aggregated form of insulin was found to be 23.1 ± 1.8 U/mg (3 determinations) by the epididymal fat pad method [9].

The insulin binding experiments were carried out in an incubation medium containing 40 mM sodium phosphate buffer (pH 7.0), 0.1% bovine serum albumin, 0.9% NaCl, 0.6 mM thiomersalate, and plasma membranes. The total volume of the reaction mixture was 0.5 ml for the saturation studies. Temperature equilibration was reached in the incubation medium containing plasma membranes, and the binding reaction was initiated by the addition of ^{125}I-insulin. Constant mixing was maintained throughout the binding period.

Upon termination of the binding reaction with 5% TCA (final conc.) the test tubes were placed immediately into an ice bath. Bound and free insulin were separated by filtration, under negative pressure, through a Millipore filter apparatus fitted with a type A glass fibre filter (Gelman, California). Two 2 ml washings by the incubation medium were sufficient to wash out more than 97% of the free iodinated insulin from the precipitated membranes retained on the filter in the control experiments. The filters were then placed into 10 ml scintillation cocktail and counted to less than 1% error [7]. The amount of ^{125}I-insulin taken up non-specifically by the precipitated plasma membranes was determined by simultaneously injecting a known quantity of insulin and plasma membranes into 5% TCA. The precipitated mixture was treated as described above, and used as a zero-time control. The controls consistently represented less than 15% of the bound values.

Due to the drastic conditions used to terminate the binding reaction, another assay method was devised to verify the method described above. Plasma membranes were incubated in 0.5 ml sodium phosphate buffer (pH 7.0) in the presence of ^{125}I-insulin contained in Eppendorf microcentrifuge tubes. The binding reaction was terminated by rapid centrifugation of the tubes in an Eppendorf microcentrifuge for 3 min at 15,000 *g*. The medium was aspirated carefully and the pelleted membranes with bound insulin were taken up into 0.1 ml water and counted for radioactivity. The amount of radioactivity taken up into the pellet non-specifically was less than 5% of the radioactivity of the specifically bound insulin. A comparison of the two methods for detecting insulin binding is shown in table 1.

Table 1
Comparison of methods of assay of ^{125}I-insulin binding to plasma membranes.

Time of assay (min)	Methods	
	TCA ppt.	Centrifugation
	(ng ^{125}I-insulin/mg)	
30	4.30 ± 0.29	4.16 ± 0.55
45	4.98 ± 0.38	4.84 ± 0.43

Conditions: see sect. 2. 0.5 ml medium contained 80 μg protein and 10 mg ^{125}I-insulin at 37°. Assays were carried out in triplicate at sub-saturating insulin concentrations. Values are ± S.E. of the mean.

3. Results

The complex nature of the time course of insulin binding is shown in fig. 1. The initial binding response at 37° reaches a peak in approximately 30 sec (Phase I). After the initial binding of insulin to the plasma membranes, there is a release of radioactivity, and then a slower binding of insulin (Phase II). This oscillatory binding response is observed at selected temperatures from 4°–41°, and is reproducible within ±10% (±S.E. of the mean) in each plasma membrane preparation used. At lower temperatures (fig. 1) the amplitudes of insulin binding are decreased and the periods of Phases I and II are increased. After approximately 10 min,

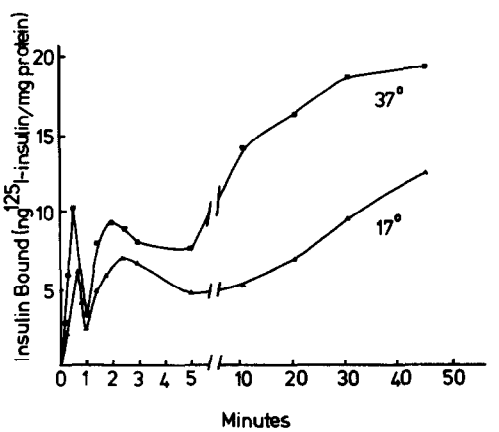

Fig. 1. Kinetics of ^{125}I-insulin binding to plasma membranes. Conditions: 3.5 ml incubation medium (see sect. 2), 0.41 mg protein, 0.25 μg ^{125}I-insulin (2 μCi/μg). Aliquots were taken from rapidly stirring mixture at indicated times and injected into 5% TCA.

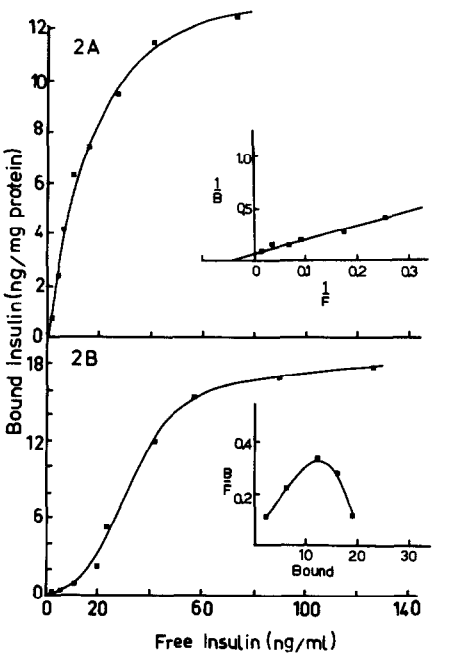

Fig. 2. Saturation binding curves of ^{125}I-insulin to plasma membranes. Conditions: the incubation medium contained 90 μg protein and ^{125}I-insulin (2 μCi/μg) at 37°. Each point is represented by the average of two determinations. All mixtures were shaken vigorously upon the addition of insulin.

Table 2
Effect of non-labelled insulin on ^{125}I-insulin binding to plasma membranes.

Non-labelled insulin (ng/ml)	cpm bound/assay
0	1364 ± 72
2.5	1002 ± 55
10.0	622 ± 27
20.0	443 ± 31
30.0	365 ± 7

Conditions: see fig. 2. Incubation time was for 45 min. 10 μg/ml ^{125}I-insulin and non-labelled insulin (24.1 U/mg) were added simultaneously. All assays were done in triplicate at sub-saturating levels of insulin. Values are ± S.E. of the mean.

there is another binding phase (Phase III) which proceeds until saturation is apparently reached. The rate of binding and the total amount of insulin bound in Phase III is also a function of temperature (fig. 1).

Saturation binding curves (fig. 2) of Phases I and II show that there is a qualitative difference between the two phases. Phase I binding (fig. 2A) follows a simple Michaelis-Menten type of saturation, and a Lineweaver-Burk plot (fig. 2A, inset) results in a linear curve. The resulting dissociation constant is 3.4×10^{-9} M. On the other hand, Phase II shows a sigmoidal saturation curve, and a Scatchard plot of the data (fig. 2B, inset) results in a concave curve suggesting a co-operative interaction between two insulin binding sites [11], or an association interaction between two molecules of insulin. A Hill plot of Phase II binding results in a coefficient of $n = 1.92$. confirming the co-operative interaction deduced from the Scatchard plot. The corresponding apparent dissociation constant is 6.1×10^{-9} M.

Controls were undertaken to show that the binding results presented in figs. 1 and 2 were the result of actual insulin binding, and not due to an isotope effect of ^{125}I-insulin. The addition of increasing amounts of non-labelled insulin (24.1 U/mg) resulted in a decrease in the total amount of radioactivity found in the precipitated pellet (table 2). Also, results similar to fig. 2 were obtained if non-labelled insulin instead of ^{125}I-insulin was used to alter the initial insulin concentrations in the medium. The TCA soluble radioactivity of the iodinated preparations used was less than 2%, and this material was incapable of binding to plasma membranes. In the plasma membrane preparations used there was no increase in the TCA soluble material during incubation, and therefore this material did not contribute to or obviate the binding results.

4. Discussion

The complex nature of insulin binding to plasma membranes should not be surprising, since it has been shown that the action of insulin on whole and fragmented tissues is multifold [10]. Similar insulin binding kinetics have been observed in adipose tissue [1]. Crofford has suggested [1] that the initial binding response of less than 1 min is due to the interaction of insulin with a specific membrane-bound receptor in the adipocytes.

The low apparent dissociation constants for Phases I and II indicate that the binding of insulin to plasma membranes is relatively tight, even though there are several periods of release. It is much too early to determine if the binding of insulin is of a covalent nature, but an analysis of the effect of temperature on the binding responses of Phases I and II suggest an activation energy of at least −10 kcal/mole [7]. For a ligand the size of insulin, this energy might be accounted for by multiple side-chain interactions on the protein surface.

The biphasic kinetics and the sigmoidal character of insulin binding during Phase II reflects the strong possibility that there are two receptor-sites for insulin that act in concert with each other in a co-operative manner [11]. On the other hand, a possible mechanism implicating the aggregation state of insulin might be suggested [12]. Physical studies on the aggregation of insulin have shown that insulin may exist as a dimer or higher aggregate at physiological pH [13]. X-ray crystallographic analysis of insulin has shown that the two monomers of the insulin dimer have different conformations, and that a strained condition may exist in this form [14]. A 'priming' [15] or activation of the insulin receptor-site (Phase I) may require a specific form of insulin, i.e. the dimer. Since the various aggregated forms of insulin are in equilibrium with each other [13], this would cause a sigmoidal saturation binding curve if both dimer and monomer were able to react at the activated receptor-site [12].

Further experiments are in progress to determine the requirements for maximal stimulation and inhibition of insulin binding to rat liver plasma membranes in conjunction with the effect of insulin on biological functions of the membrane.

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