Biochimica et Biophysica Acta, 600 (1980) 931—938

BBA 78896

TRANSFER OF INSULIN RECEPTORS AND OF GLUCOSE TRANSPORT-INDUCING PROTEINS ONTO PHOSPHOLIPID VESICLES

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(Received January 16th, 1980)

Key words: Glucose transport; Insulin receptor; Phospholipid vesicle

Summary

Insulin receptors and glucose transport-inducing proteins have been extracted from rat liver membranes onto positively charged lipid bilayer vesicles. The extraction was carried out during the incubation of the vesicles with lipid vesicles caused an overall enhancement of specific insulin binding and of glucose transport inducement. The latter has been inferred from the oxidation rate of transported glucose through a spherical bilayer membrane entrapping the oxidizing glucose oxidase. Glucose transport is not enhanced by insulin binding, indicating that the two functions become dissociated when the proteins are transferred from the plasma membrane onto the bilayer vesicles.

Introduction

During the past few years, considerable progress has been made in the study, identification, isolation and purification of a variety of membrane-localized receptors. The direct evidence for the localization of insulin receptors on the plasma membrane of the cell has been obtained from extensive binding studies that utilize ^{125}I-labelled insulin and isolated cells or microsomal membrane preparations. The insulin receptor, despite being an integral part of a biological membrane, does not require the membrane environment for recognition of insulin.

The insulin receptor was quantitatively extracted in soluble form from liver and fat cell membranes with the non-ionic detergent, Triton X-100 [1,2], and from lymphocytes with the detergent, NP-40 [3]. Small amounts (less then 10% of the total present) of insulin receptor can be solubilized by simple

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shaking of liver or fat cell membranes in the absence of detergents in neutral buffer or distilled water [4]. In the absence of detergents, however, these structures tend to precipitate after they are concentrated to characterize their properties. A reconstituted system of lipids and receptors is expected to be stable in solution. The question was whether a reconstitution process without involvement of detergents is feasible. To answer this question we have investigated the interaction of plasma membrane proteins contained in membrane fragments with liposomes in neutral media.

The purpose of this investigation was to determine the capacity of the lipid vesicles, depending on their composition, to extract the membrane proteins and to find out whether such extraction is selective. To reach this aim we measured the insulin-binding properties of the membrane proteins transferred onto the liposomes and their capacity to induce glucose permeability as well as the effect of added insulin on the glucose transport.

Experimental Procedure

Materials. Glucose oxidase (highly purified grade) was obtained from P. and L. Biochemicals, Milwaukee, WI, U.S.A. Egg lecithin (grade I) and phosphatidylserine were obtained from Lipid Products (Nutfield, U.K.). Cholesterol was purchased from B.D.H. Chemicals, Poole, U.K.; stearoylamine from K and K Laboratories, Plainview, NY. Insulin (crystalline; 23—35 I.U./mg) from Fluka AG Buchs, Switzerland, and ^{125}I-labelled insulin from New England Nuclear with specific activity 84.88 μCi/μg. Triton X-100 from Sigma Chemicals Co., St. Louis, MO. All other reagents were of analytical grade.

Preparation of liposomes. Neutral bilayer liposomes were prepared from egg phosphatidylcholine and cholesterol (molar ratio 7 : 2). To obtain negatively or positively charged vesicles, we supplemented the neutral lipids with negative phosphatidylserine or positive stearoylamine lipids. The molar ratio or phosphatidylcholine, cholesterol and phosphatidylserine or stearoylamine, respectively, was 7 : 2 : 1 [5].

Preparation of liver plasma membrane fragments. The liver plasma membranes from Sprague-Dawley rats (140—180 g) were prepared from homogenates by differential centrifugation in 0.25 sucrose solutions according to the method of Ray [6]. Protein was determined by using the method of Lowry et al. [7] using bovine serum albumin as a standard.

Extraction of plasma membrane proteins by lipid vesicles. The vesicles prepared from different lipid mixtures were incubated with plasma membrane preparations for 1 h at 37°C with continuous shaking. The reaction mixture was centrifuged at 5000 rev./min for 15 min. The supernatant containing small liposome-bound plasma membrane was kept and the pellet was dispersed and reincubated with a new portion of liposomes. The reaction mixture was then separated under the same conditions as described above. Only very small (less than 300 Å) membrane fragments besides membrane proteins could remain attached to the vesicles in the clear suspension after centrifugation at 5000 rev./min.

The amount of protein attached to the liposome was determined using the method of Lowry et al. [7].

Binding experiments. The methods employed in the binding studies have been previously described in detail [8]. In brief, ^{125}I-labelled insulin was incubated with plasma membrane or with liposomes containing the extracted membrane proteins in phosphate buffer, pH 7.8, containing 1% bovine serum albumin. The membrane-bound hormone was separated by filtration and washing on EGWP Millipore filters (0.22 μm) preincubated with 10—30% bovine serum albumin solution. The hormone retained on the filter was counted in an automatic γ-counter (Packard). Specific binding was taken to be the amount of hot insulin bound and then released by excess of cold insulin. The non-released hot insulin was considered as non-specifically bound. The specific binding of insulin to plasma membrane and to liposomes containing membrane proteins was analysed by Scatchard plots to calculate the number of receptor sites and the apparent dissociation constant K_d [9].

Entrapment of glucose oxidase. Entrapment of glucose oxidase into vesicles and the determination of free and total enzymic activity were described by us in a previous work [5]. Briefly, the lipid mixture at the desired composition (the most effective was the one containing lecithin, cholesterol and stearoylamine at a molar ratio of 7 : 2 : 1 was cosonicated with the glucose oxidase. Glucose oxidase was then removed from the external solution by successive washing with solutions of high salt concentrations using an Amicon ultrafiltration cell model 12 with Diaflo membrane XM300. The enzymic activity of the residual outer and of the inner enzyme acting on the glucose transported through the lipid bilayer membrane was determined by monitoring the initial uptake rate of oxygen using a Clark O_2 electrode Model YSI (Yellow Spring Instruments). The total glucose oxidase activity was determined after liberation of the entrapped enzyme by incubation with a 1% solution of Triton X-100 at room temperature for 30 min.

Measurement of the inducement of membrane permeability to glucose. Samples of vesicles entrapping glucose oxidase were incubated with different amounts of plasma membrane fragments or liposomes containing plasma membrane proteins for different periods of time. The enzymic activity of the entrapped glucose oxidase in the reaction mixture and in controls free of membrane proteins was determined as described.

To measure the effect of insulin on the induced glucose transport, the above reaction mixtures were incubated with different amounts of insulin for various periods of time and enzymic activity was checked again.

Results

Extraction of plasma membrane proteins by lipid vesicles

The protein content of the membrane fragments obtained from 10 g rat liver was about 10 mg. The saturation values of the specific adsorption of insulin, as obtained from the Scatchard plots presented later, varied for two different membrane preparations between 3 and 5 ng insulin per mg plasma membrane proteins. Since the molecular weight of insulin is 6000 there is about one adsorption site equivalent per $2 \cdot 10^9$ and $1.2 \cdot 10^9$ g of membrane protein, respectively. Neutral and negatively charged lipid vesicles did not extract any appreciable amount of proteins from the membrane fragments.

24 mg of positively charged lipid vesicles incubated with 1 mg plasma membrane fragments in 6 ml of 0.01 M phosphate buffer at pH 7.2 extract about 25% of the membrane proteins. The maximal specific insulin binding of the lipid vesicles with extracted membrane proteins from the two membrane preparations amounted to 26 and 70 ng insulin/mg protein. This corresponds to one binding site equivalent per $2.3 \cdot 10^8$ g and $8.6 \cdot 10^7$ g membrane proteins, respectively. Thus, the insulin-binding capacity of the extracted proteins increased by factors of 9 and 14. This is surprising, since even if all the receptors were among the 25% of the extracted protein the binding capacity should increase only by a factor of 4. Moreover, the insulin-binding capacity of the membrane fragments from which the proteins, including the insulin receptor, have been extracted, is not diminished. It follows from here that the lipid treatment of the membrane fragments reveals hidden insulin-binding sites. This is illustrated in Fig. 1 where the total and the non-specific adsorption curves are expressed per mg protein of plasma membrane fragments (Fig. 1a), per the protein on the lipid vesicles extracted from the 1 mg protein on the plasma membrane; (Fig. 1b) and per the remaining protein from the 1 mg in the membrane fragment (Fig. 1c). In Fig. 1a also the sum of the binding curves to the extracted and the remaining protein from the initial 1 mg plasma protein is given. It is also evident from this figure that the ratio of the non-specific binding to the extracted membrane proteins and to the lipid-treated membrane fragments has increased.

Specific insulin binding

The specific binding of ^{125}I-labelled insulin to membrane fragments and to receptors extracted from the membranes by phospholipid vesicles was determined. We have shown that the liposomes not only extract preferentially the receptors as compared to the other membrane proteins, but also enhance the overall binding capacity. The adsorption isotherms presented relate only the specific adsorption, namely, the relatively few binding sites which adsorb insulin very strongly and become saturated already in the presence of the very low concentration of the labelled insulin. Upon addition of a large excess of unlabelled insulin, most of the labelled insulin is displaced until the ratio of the labelled to unlabelled insulin becomes like that in the solution, while the number of the sites occupied by the insulin does not change any more. In contradistinction to the specific binding sites, there is an abundant number of non-specific binding sites. These may include binding sites on the liposomes and on the Millipore filters which are free from saturation, even in the presence of the large excess of the unlabelled insulin. The labelled insulin adsorbed on these sites is therefore not displaced by the excess of non-labelled insulin. Control experiments with pure phospholipid vesicles showed non-specific binding but no trace of specific binding.

In Fig. 2 the adsorption isotherms on the specific binding sites (displaced by excess of non-labelled insulin) are given for two different preparations of receptor-enriched liver membrane fragments. A series of independent adsorption experiments produced practically the same isotherms. It is evident that in one preparation the binding capacity per total protein is about twice that of the other. There was also a difference in the binding constant as obtained

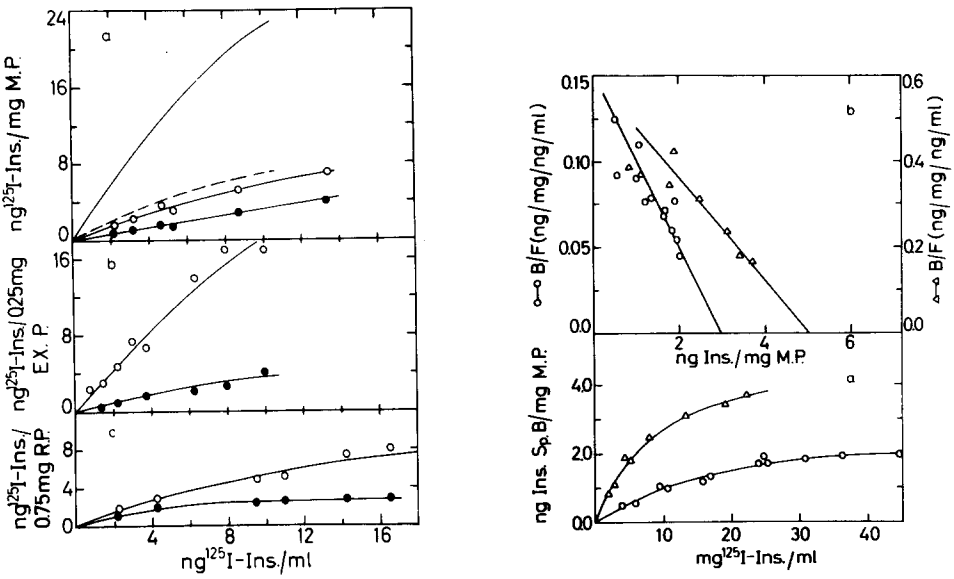

Fig. 1. Adsorption of ^{125}I-labelled insulin (^{125}I-Ins.) as a function of its concentration in solution in the absence (○) and presence (●) of excess (50 μg/ml) cold insulin. (a) Curves with symbols: adsorption of ^{125}I-labelled insulin per mg of protein in the adsorbing membrane fragments. Without symbols: sum of the adsorption on phospholipid vesicles with the protein extracted (approx. 0.25 mg) from 1 mg membrane protein (M.P.) on the fragments and of the adsorption on the fragments with the residual protein. (0.75 mg). No cold insulin added (———); excess of 50 μg/ml cold insulin added (- - - - -). (b) Adsorption isotherms of ^{125}I-labelled insulin in the presence and absence of excess cold insulin on phospholipid vesicles containing approx. 0.25 mg protein extracted (EX.P.) from membrane fragments (1 mg protein content). (c) Adsorption isotherm of ^{125}I-labelled insulin in the presence and absence of cold insulin membrane fragments with approx. 0.75 mg protein remaining (R.P.) from 1 mg after extraction with liposomes.

Fig. 2. Binding of insulin to membrane fragments. (a) Specific adsorption isotherm of ^{125}I-labelled insulin (^{125}I-Ins.) (adsorption displaced by excess of cold insulin expressed per mg of membrane protein (M.P.)) on two preparations of membrane fragments (○,△) (Sp.B., specific binding). The points are experimental. The curves are reconstructed from the straight lines of the Scatchard plot. (b) Constructed from the experimental points of the two adsorption isotherms.

from the Scatchard plot (Fig. 2b); about $3 \cdot 10^8$ l/mol in one preparation and $7.2 \cdot 10^8$ l/mol in the other. However, this difference in the determined binding constant is still within the experimental uncertainty.

The transfer of the insulin receptor from the membrane onto liposomes and bilayer vesicles depends on their composition. The most efficient in this respect were bilayer vesicles containing 70% lecithin, 20% cholesterol and 10% stearoylamine. The extracted protein was also considerably enriched with insulin receptor, as can be seen from Fig. 3. The maximal specific insulin binding per mg protein was 26 and 70 ng which is about 9 and 14 times larger than that observed in the receptor enriched membrane fragments (3 and 5 ng). The value of the binding constant obtained from the Scatchard plot is very close even though somewhat larger, $(8 \pm 1) \cdot 10^8$ l/mol and $(11 \pm 3) \cdot 10^8$ l/mol, than that of the receptors on the membrane fragments. All these values are within the spectrum of reported values [10]. In both cases we could discern only one binding constant. One has to bear in mind that the value of K_a

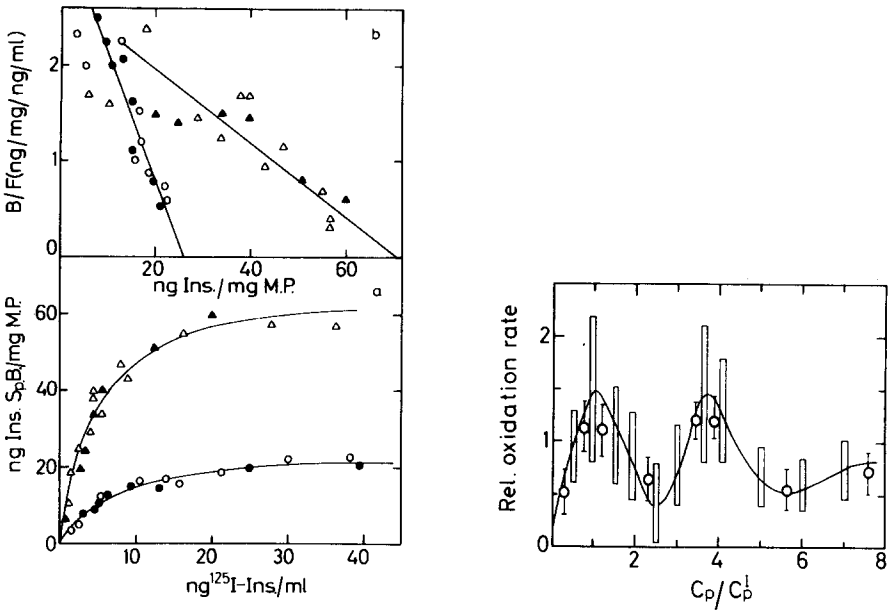

Fig. 3. Binding of insulin to membrane proteins on liposomes. (a) Specific adsorption isotherms of ^{125}I-labelled insulin (adsorption displaced by excess of cold insulin) to receptors extracted onto positively charged bilayer vesicles from two preparations of membrane fragments (○, ●, △, ▲). The adsorption is expressed per mg of membrane protein (M.P.) (Sp.B., specific binding). The points are experimental (open and filled symbols, experiments taken at different dates). The curves are reconstructed from the straight lines of the Scatchard plots. (b) Constructed from the experimental points of the two adsorption isotherms.

Fig. 4. Relative oxidation rate of glucose in the outer solution by entrapped glucose oxidase as a function of the concentration of added membrane protein, C_p, divided by its concentration at the appearance of the first maximum, C_p^I. The relative oxidation rate is defined as the fraction of the total oxidation rate by the glucose oxidase after its release from the liposomes by Triton X-100. Bars with membrane fragments, circles with membrane protein extracted onto phospholipid vesicles. C_p^I in the case of membrane fragments varied between 15 and 30 μg/ml, in the case of extracted membrane proteins onto liposomes it was 1.5 and 2 μg/ml.

obtained from the Scatchard plot is not very accurate and adsorption isotherms of a very similar shape can give K_a values differing by a factor of 2 or even more. We can conclude that lipid vesicles can extract proteins and the insulin-binding protein is extracted preferentially. As will be shown later, there is also a preferential extraction of the glucose transport-inducing protein. The functional relationship between these two proteins is still obscure. It would be useful to know whether there is any such relationship.

Inducement of glucose transport by the membrane proteins

The insulin receptor on the membrane is accompanied by the glucose-transport system. It is not clear whether the two systems are interconnected or completely independent. Selective extraction of a glycoprotein of the rat adipocyte plasma membrane was recently carried out in an attempt to identify the membrane component involved in glucose transport [11]. The stereo-specific glucose transport has then been reconstituted by incorporation of this substance into liposomes. To check the glucose transport activity we mixed the

membrane fragments or the vesicles containing the extracted membrane proteins with vesicle containing entrapped glucose oxidase. The enhancement of the oxidation of the glucose dissolved in the outer solution by the entrapped glucose oxidase was taken as a measure for the induce glucose transport.

The interactions of the different membrane and entrapped glucose oxidase preparations show qualitatively similar behaviour, however, the quantitative results are not very reproducible. In every case, the oxidation of the glucose by the entrapped glucose oxidase increased with added membrane proteins until a maximum had been reached. Then the oxidation rate decreased to a minimum and then increased again to a second maximum. A third maximum was observed in some cases. For purified membrane fragments the first maximum was reached at a membrane protein concentration between 15 and 30 μg/ml depending on the preparation and the exact lipid vesicle concentration. The lipid concentration in the different experiments varied between 1 and 2 mg/ml. In the case of membrane proteins extracted on lipid vesicles, the first maximum appeared at a protein concentration of about 1.5 μg/ml. Thus, similarly to the insulin receptor, there was also a preferential extraction of the glucose transfer-inducing proteins.

Against our expectations, addition of insulin did not enhance significantly the transport of glucose even though it has an effect of its own on the glucose transport. In Fig. 4 a combined picture of the effect of the membrane proteins on the glucose transport is given. The protein concentrations are normalized with respect to the concentration at which the first peak appears while the activity is given in arbitrary unit normalized with respect to the total activity of the entrapped glucose oxidase. The bars give the dispersion of the experimental points for eight experiments with two different preparations of purified membrane fragments. Repeated experiments with the same membrane and entrapped glucose oxidase preparations indicate that the mere way of mixing of the membrane with the vesicles containing the entrapped glucose oxidase affects the results quite significantly. Accounting for the much higher activity of the extracted membrane proteins on the vesicles, their effect on the glucose transport as presented on the normalised scale (circles) is comparable to that of the purified membrane fragments.

It is very difficult to interpret these results. As a working hypothesis, we can consider the possibility that the proteins clamp together when interacting with the liposomes. They may be active when fairly dispersed but they may become inactive in the aggregated form. Thus, the glucose transport increases at the beginning with the concentration of the membrane which adsorbs as individual molecules or small aggregates on the vesicles containing the entrapped glucose oxidase. Since, however, the affinity of the further added proteins to the already adsorbed ones is much larger than to the pure lipid, the protein nuclei will grow until inactive protein aggregates are formed. Only above a certain size of these aggregates does their attraction to additional protein molecules diminish and the added membrane proteins disperse over the bare lipid vesicles to form new active nuclei. Such a process can go on only under non-equilibrium conditions and it is likely not to be very reproducible. Moreover, the transport is passive and may even be non-specific to glucose but to a whole number of molecules of the right size and chemical nature.

Discussion

Reconstitution of the functions of the membrane proteins by their incorporation into lipid matrices is usually used for studying the function-structure relationship and the specific lipid requirement of the proteins for their function. The insulin receptor dissolves to some extent in water and it also binds insulin in the aqueous solution. However, the affinity of the receptor to insulin may depend, besides on the nature and source of the receptor, also on the binding environment. The quantitative aspects of insulin reaction with its receptor have been considered in at least seven laboratories by ten different investigators using a variety of preparations of fat and liver cells and lymphocytes. The measured affinity constants vary from as high as $2 \cdot 10^{10}$ to as low as $1.3 \cdot 10^{5}$ M^{-1}. Results indicate that the population of insulin receptors in the purified liver membrane can indeed be considered heterogeneous with respect to affinity for insulin [10]. Binding of insulin per se is not the sole function of the receptor. The insulin binding to cell membrane is related to the glucose transport. The functional relationship follows, presumably, from the structural relationship between the insulin receptor and the glucose system in the membrane. This structural relationship seems to be abolished when the insulin receptor together with the glucose transport system are extracted onto bilayer vesicles either directly or via other liposomes. At least the lack of enhancement of glucose transport by added insulin, as inferred from the glucose oxidation by the entrapped glucose oxidase, points in this direction. Here the glucose transport system and the insulin receptor are dislocated from their native sites and go out of mutual register. At the same time, extraction of the membrane proteins by the positively charged lipid vesicles augments their insulin binding and their glucose transport induction. This may be caused by the preferential extraction of the insulin receptor and of the glucose transport system and by their activation.

The fact that the insulin binding of the membrane fragments is not diminished after the receptor extraction suggests activation or revealing of hidden receptors. The same conclusion can be drawn with respect to the glucose transport system.

The efficiency of different lipids to activate the insulin receptors and the glucose transport system is not clear as yet and is a subject for further investigation.

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