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DISTRIBUTION OF INSULIN RECEPTORS AMONG MOUSE LIVER ENDOMEMBRANES

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Summary

Specific binding of insulin to highly purified preparations of rough endoplasmic reticulum, Golgi apparatus, and plasma membrane of mouse liver was determined. ^{125}I-labeled insulin bound maximally to the plasma membrane in radio-receptor assays. Golgi apparatus fractions exhibited binding 10—20% that of plasma membrane and rough endoplasmic reticulum exhibited only 1—2% of plasma membrane binding. Binding was proportional to membrane concentration and dose vs. response curves were very similar for the different fractions. Scatchard analysis of the insulin binding data for the plasma membrane and Golgi apparatus fractions showed curvilinear plots yielding similar apparent binding affinities (0.9 and $3.0 \cdot 10^8$ M^{-1}, respectively). Purity of the isolated endomembranes was analyzed by morphometry and ($Na^+ + K^+ + Mg^{2+}$)-ATPase and these preparations displayed less than 1% contamination by plasma membrane. These findings provide important confirmation of the presence of insulin receptors in Golgi apparatus membranes comparable to those located on the plasma membrane. Finally, the present study did not allow us to verify the existence of insulin receptors in the endoplasmic reticulum.

Introduction

We reported that NADH-indophenol reductase was inhibited by insulin in not only plasma membrane but also internal membranes of mouse liver [11,

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16]. In order to determine whether the inhibition of NADH-indophenol reductase by insulin observed in the internal membranes was mediated through hormone binding to its receptor, the distribution of insulin receptors in these endomembranes was investigated.

The binding of insulin to its specific receptors has been clearly demonstrated in highly purified preparations of rodent liver plasma membranes [23]. Although the exact mechanism of insulin action in the cell is poorly understood, the first step in hormone-receptor interaction that leads to a biological response seems to take place at the plasma membrane.

Insulin-binding activity has been reported previously for both the Golgi apparatus [2], and the endoplasmic reticulum [13] of rat liver. Since the significance of these studies depends upon the purity of the isolated endomembrane fractions with regard to plasma membrane contamination, it was important to confirm the existence of insulin binding activity in similar preparations of known high purity from another source. Additionally, the presence of hormone receptors, such as insulin binding sites, on internal cytomembranes is of significance to concepts of surface membrane origin and biosynthesis. One hypothesis, that of membrane flow [8], would predict that hormone receptors might be inserted into plasma precursors, such as those provided by the Golgi apparatus vesicles, prior to their arrival at the plasma membrane [20].

Competitive binding techniques have been successfully employed in the characterization of insulin receptors in vitro [9]. These techniques were used in this study to determine the subcellular distribution of insulin binding sites in mouse liver.

Materials and Methods

The labeled and unlabeled insulin used in this study were generously provided by the Diabetes Branch, National Institutes of Health, Bethesda, MD. The standard binding assay mixture included 50 μl each of: (a) tracer, porcine ^{125}I-labeled insulin, 150—250 pg/ml (180 Ci/g insulin, approximately 10 000 cpm at 85% counting efficiency); (b) receptor source, 100—500 μg membrane protein/ml, and (c) buffer, Krebs-Ringer phosphate buffer, pH 7.4, containing 1.0 mg/ml final concentration of bovine serum albumin and supplemented with unlabeled peptides, final concentrations as specified. The assay mixture was incubated in 0.4 ml microfuge tubes at 4°C for 18 h, conditions chosen to minimize hormone and receptor degradation and insure steady-state equilibrium [23]. After incubation, duplicate tubes were centrifuged in a Beckman microfuge for 1 min at room temperature and the supernatant aspirated with a needle attached to a suction. Tube tips containing the membrane pellets were cut off and the radioactivity determined (Nuclear Chicago autogamma counter). Binding was calculated as radioactivity of pellet/total radioactivity of the reaction mixture. Degradation of the hormone was measured by trichloroacetic acid precipitability and under the experimental conditions used, was found to be less than 5% (unless otherwise stated).

Purified plasma membrane [28], Golgi apparatus [19], and rough endoplasmic reticulum [4,18] isolated from mouse liver as described previously for rat liver supplied the receptor sources for binding experiments. Protein was

determined by the method of Lowry et al. [17] with bovine serum albumin as a reference standard. Morphometric estimations of membrane components were made by using the method of Loud [15]. The assay conditions for (Na^+ + K^+ + Mg^{2+})-ATPase were as described by Emmelot et al. [5].

Results

The relative specific binding capacity of the three mouse liver membrane fractions, i.e. plasma membrane, Golgi apparatus, and rough endoplasmic reticulum, increased in direct proportion to increasing protein concentration (Fig. 1). Insulin degradation measured by trichloroacetic acid precipitability was less than 5% for Golgi apparatus and endoplasmic reticulum. Degradation (15%) was evidenced by plasma membranes only at the highest protein concentration.

The inhibition of ^{125}I-labeled insulin binding by increasing concentrations of unlabeled insulin (Fig. 2) showed very similar dose vs. response curves in all of the membrane fractions. Half-maximal inhibition occurred between 40 and 50 ng/ml of unlabeled insulin. When the total binding/mg protein of the different fractions was compared, the rough endoplasmic reticulum was found to yield 4% and the Golgi apparatus 17%. Specific binding was determined as the difference between the total amount of ^{125}I-labeled insulin bound minus the amount of ^{125}I-labeled insulin bound in the presence of an excess of unlabeled insulin (10 μg/ml). Specific binding of the endomembranes as compared to the plasma membrane gave the following results: rough endoplasmic reticulum specifically bound 2% that of plasma membrane, and Golgi apparatus specifically bound 14% that of the plasma membrane fraction.

Scatchard plots [14,26] of insulin binding to plasma membrane and Golgi apparatus (Fig. 3) yielded apparent binding affinities of 0.9 and 3.0 · 10^8 M^{-1}, respectively, calculated from the slope of the steepest portion of the curve. The number of receptor sites calculated for the Golgi apparatus was approximately

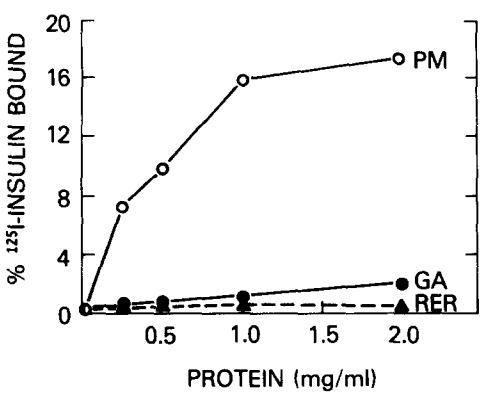

Fig. 1. Specific binding of ^{125}I-labeled insulin to isolated fractions of plasma membrane (PM), Golgi apparatus (GA), and rough endoplasmic reticulum (RER) as a function of membrane protein concentration in mg/ml. Fractions were incubated at 4°C for 18 h with ^{125}I-labeled insulin (150 pg/ml). Hormone degradation was 5% or less except at the highest concentration of plasma membrane protein where degradation of 15% was observed.

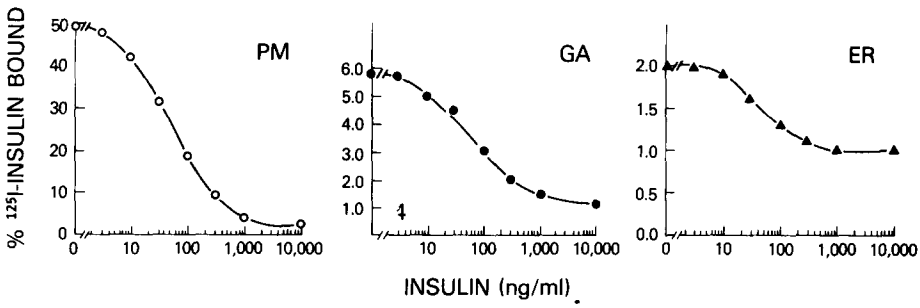

Fig. 2. ^{125}I-labeled insulin binding to plasma membrane (PM), Golgi apparatus (GA), and rough endoplasmic reticulum (ER) fractions of mouse liver. Percent of total ^{125}I-labeled insulin bound is plotted against the final concentration of unlabeled insulin (ng/ml). Incubations were for 18 h at 4°C utilizing 0.3 ng of plasma membranes and Golgi apparatus/ml and 1.6 ng of rough endoplasmic reticulum/ml.

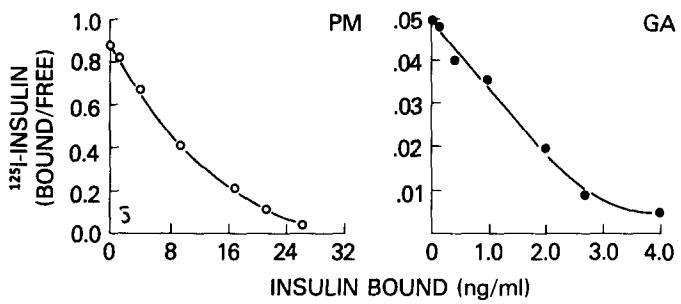

Fig. 3. Scatchard analysis of ^{125}I-labeled insulin binding to the plasma membrane (PM) and Golgi apparatus (GA) of mouse liver. The bound/free ^{125}I-labeled insulin is plotted as a function of bound hormone. Non-specific binding, which has been subtracted, was equal to 2.3% of the total radioactivity added with plasma membranes, and 1.1% with Golgi apparatus.

TABLE I

PURITY OF CELL FRACTIONS FROM MORPHOMETRY

Units are membrane intercepts/100 intercepts ± S.D. for the actual preparations analyzed for insulin binding. n.d., none detected. Details of the procedure and the data necessary to establish that conditions for random sampling are fulfilled are provided by Morré et al. [22].

Component present	Fractions analyzed		
	Endoplasmic reticulum	Golgi apparatus	Plasma membrane
Endoplasmic reticulum	97.0 ± 0.0	4.0 ± 4.0	0.4 ± 0.5
Golgi apparatus	0.1 ± 0.1	93.0 ± 4.0	n.d.
Plasma membrane	0.4 ± 0.1	0.4 ± 0.1	97.6 ± 1.2
Mitochondria	0.3 ± 0.2	0.4 ± 0.3	1.0 ± 0.7
Microbodies	0.1 ± 0.0	<0.1	n.d.
Lysosomes	0.1 ± 0.0	<0.1	n.d.
Unidentified	1.3 ± 0.1	1.3 ± 0.7	1.0 ± 0.2

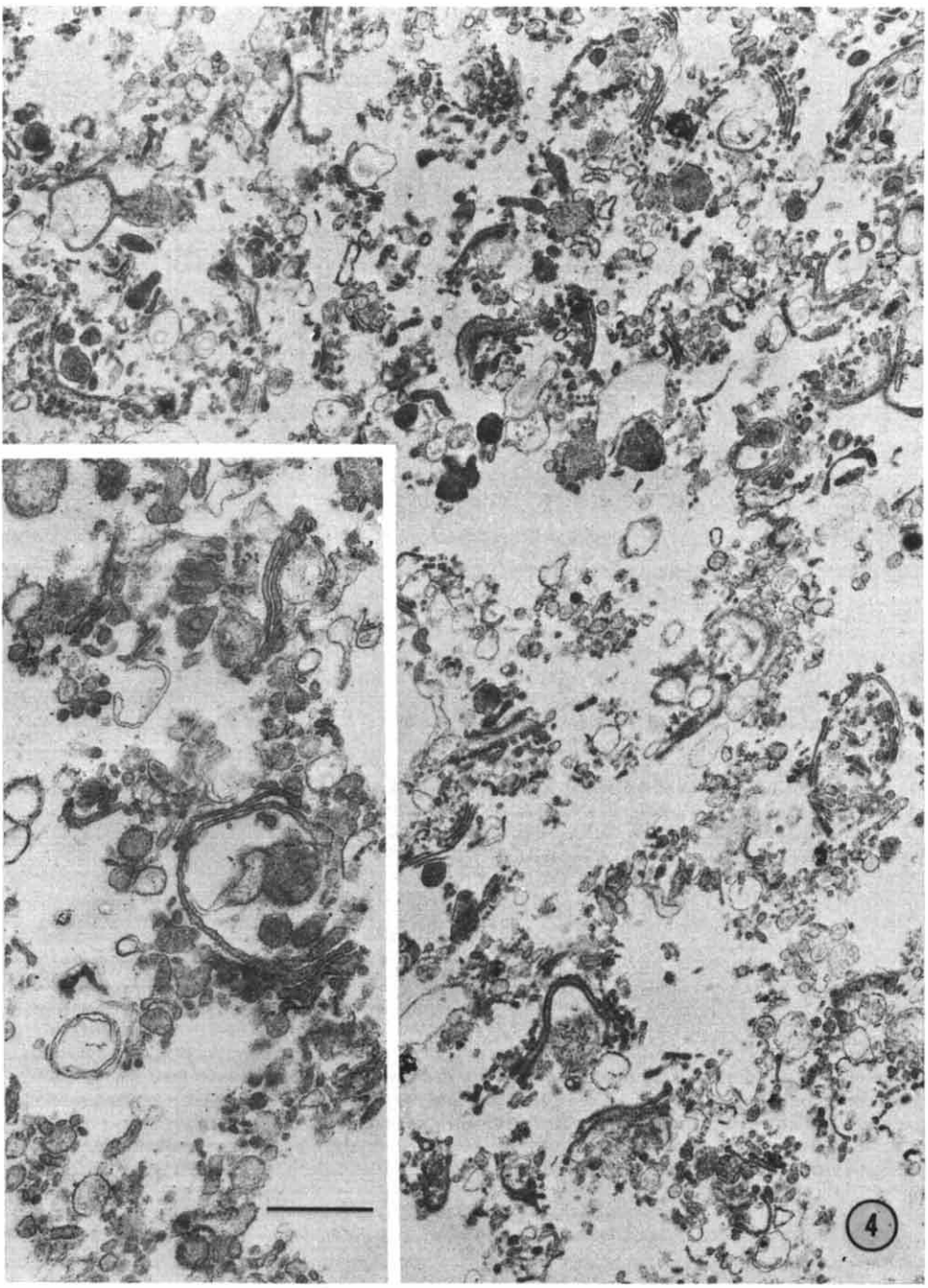

Fig. 4. Representative electron micrographs of a Golgi apparatus fraction utilized in the binding studies described. The preparations contain numerous stacks of cisternae (dictyosomes). Less than 1% of the total membrane is contributed by plasma membrane vesicles. Scale marker = 1 μm. Inset is at higher magnification. Scale marker = 0.5 μm.

TABLE II

PURITY OF CELL FRACTIONS FROM ACTIVITIES OF $(Na^+ + K^+ + Mg^{2+})$-ATPase

Cell fraction	μmol P_i liberated/h per mg protein (± S.D.)	% of plasma membrane
Total particulate	1.0 ± 0.4	7
Golgi apparatus	0.2 ± 0.6	1
Endoplasmic reticulum	<0.2	<1
Plasma membrane	13.9 ± 2.2	100

10% of the number found for the plasma membrane. Fraction purity as determined by electron microscope morphometery (Table I) ranged from 97% for endoplasmic reticulum to 93% for Golgi apparatus (Fig. 4). Plasma membrane contamination of these two fractions was less than 1% as evidenced both from morphometry (Table I) and specific activity of $(Na^+ + K^+ + Mg^{2+})$-ATPase (Table II). The enrichment of the activity in mouse liver plasma membrane was 14-fold relative to the total particulate fraction corresponding to a 22-fold enrichment relative to the starting homogenate.

Discussion

In the present study, we have presented evidence for the existence of insulin receptors on Golgi apparatus of the mouse hepatocyte. When specific binding of insulin to the different membrane fractions was studied at the same protein concentration, Golgi apparatus displayed 10—20% of the plasma membrane binding capacity and rough endoplasmic reticulum showed only 1—2%. Based on morphometric determinations of fraction purity the contamination by plasma membrane in both Golgi apparatus and rough endoplasmic reticulum preparations was not greater than 1%; in addition, the presence of unidentified membrane components was less than 2% in these fractions. As a second criterion of fraction purity, $(Na^+ + K^+ + Mg^{2+})$-ATPase was chosen. This activity was previously reported to be low or absent from rat liver Golgi apparatus [6] and provides a relatively specific enzymatic marker for the hepatocyte plasma membrane. Again plasma membrane contamination was found to be in the order of 1% or less. Therefore, the insulin binding observed by Golgi apparatus could not be accounted for by plasma membrane contamination and does indicate the presence of insulin receptors on these intracellular membranes. The very low levels of insulin binding by rough endoplasmic reticulum fractions may be intrinsic or possibly be contributed by plasma membrane contamination.

The insulin receptors of the Golgi apparatus were extraordinarily similar to the cell surface receptors for all parameters investigated, i.e. half-maximal inhibition of binding by unlabeled hormone (a measure of receptor affinity), and Scatchard plot analysis as previously described [9,13,23].

Our study confirms other reports of insulin binding to subcellular fractions of liver. Bergeron and coworkers [2,3,24,25] have demonstrated insulin binding sites in Golgi apparatus fractions of rat liver which are present to the same extent [2] as those in our isolated Golgi apparatus. Kahn [13] has observed

insulin binding to fractions of rough endoplasmic reticulum of rat liver separated by density gradient centrifugation.

The reconciliation of our findings of such a low level of insulin binding to the rough endoplasmic reticulum, whereas insulin inhibits NADH-indophenol reductase activity to a considerable extent in this fraction [11] remains unexplained. Whether the insulin inhibition is unrelated to hormone-receptor binding or whether endoplasmic reticulum binding may be revealed only under certain experimental conditions remains to be investigated. Even if insulin receptors in endoplasmic reticulum do exist, these receptors may be immunologically different from those located on the plasma membrane as suggested by Goldfine et al. [12].

At present, correlation of binding to the insulin receptor and function has been established in intact cells, i.e. thymocytes [27], peripheral mononuclear cells (mainly lymphocytes) isolated from obese patients [1], and other human cell lines in vitro [10]. In addition, regulation of the insulin receptor by insulin has been observed in mouse liver plasma membrane [23]. These studies have shown that the cell surface receptor is a variable quantity whose control is under diverse regulatory mechanisms. The question of where the insulin receptor originates might be answered by the phenomenon of membrane flow and differentiation. These two processes, when combined, may account for a contribution of endomembrane-derived insulin receptors to the surface membranes of hepatocytes. Membrane flow, the physical transfer of membranes from one cell component to another, has been evidenced from studies including short-term labeling and turnover of membrane proteins, kinetic analysis of electron transport chain constituents, and early drug-induced changes in enzymatic activities [7]. Membrane differentiation is evidenced from biochemical and morphological studies which show selective transfer by a gradual conversion of membranes from endoplasmic reticulum-like to plasma membrane-like across the stacked cisternae of the Golgi apparatus [21]. With insulin receptor biosynthesis, one might hypothesize a contribution from the rough endoplasmic reticulum in the synthesis of the receptor which is then transferred to the Golgi apparatus, and subsequently to the cell surface. The immunological characteristics of the receptor may change, however, during synthesis and transfer [12].

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