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Relationship of Binding to Internalization of ^{125}I-Insulin in Isolated Rat Hepatocytes

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Summary. By quantitative electron microscopic autoradiographic technique, we have previously shown that ^{125}I-insulin initially localizes to the plasma membrane of isolated rat hepatocytes and is subsequently internalized in a limited region of the peripheral cytoplasm. In the present study, we have shown that when cells are incubated at 20 °C, steady state binding is reached by 60 minutes and maintained up until 120 minutes of incubation while at 37 °C steady state binding is reached by 10 minutes and maintained for 30 minutes. Under both of these conditions, internalization of the labelled material occurs as a constant function of the binding. These data suggest that under normal conditions the binding of the ligand is an important rate limiting determinant of the internalization process.

Key words: Insulin receptor, internalization, endocytosis, hepatocytes, binding, insulin.

The initial interaction of ^{125}I-insulin with hepatocytes occurs through specific, saturable receptors on the surface of the plasma membrane. More recently, it has been demonstrated in isolated hepatocytes [1, 2, 3, 4] and in hepatocytes from intact liver [5, 6] that the interaction of insulin with its cell surface receptor results in the internalization of the ligand. While initial binding is primarily controlled by the affinity of insulin for the receptor (K) and the number of receptor sites (R_o), the factors controlling internalization are unknown. In the present study, we have investigated the relationship between the binding of the hormone and its internalization.

Materials and Methods

Cells and Reagents

Hepatocytes were isolated from normal 6 to 8 weeks old Wistar rats fed ad libitum using a modification of the method described by Seglen [7]. ^{125}I-insulin was prepared at a specific activity of 250 μCi/μg by a modification of the chloramine-T-method [8]. The labelled insulin was purified by filtration on G-50 Sephadex at 4 °C prior to each experiment.

Incubation Conditions

Hepatocytes at a final concentration of 1×10^6 cells/ml were incubated in duplicate in 0.5 ml of modified Krebs Ringer bicarbonate (KRB) (pH 7.7), containing 25 mg/ml bovine serum albumin (Fraction V) and 0.8 mg/ml of bacitracin, with 5×10^{-10}mol/l ^{125}I-insulin at 20° and 37 °C for varying periods of time. Bacitracin was added in order to prevent insulin degradation in the medium during exposure to hepatocytes [9]. Identical incubations were carried out in the presence of 2.7×10^{-5}mol/l unlabelled insulin to determine non-specific binding (i. e. cell associated radioactivity in the presence of an excess of unlabelled hormone). At the times indicated, 1 ml of chilled buffer was added to each tube, immediately followed by centrifugation for 20 seconds at 50 × g. The supernatant was discarded and the cell pellet quickly resuspended in 1 ml of chilled buffer and centrifuged for 90 seconds at about 500 × g in a Beckman plastic microfuge tube. Cell pellets were further washed (without resuspension) by chilled buffer containing 100 mg/ml of sucrose. At the end of the wash procedure, 4% glutaraldehyde in 0.1mol/l phosphate buffer was added to each cell pellet and allowed to fix for 4 h at room temperature. The glutaraldehyde was then aspirated and replaced with 0.1 mol/l phosphate buffer pH 7.4 until further processing. The radioactivity in the pellet was then determined in a gamma counter.

Preparation for Electron Microscopy and Autoradiography

After three successive washings in phosphate buffer, the cell pellet was post-fixed in 0.1 mol/l osmium tetroxide pH 7.4 for 2 hours at room temperature, dehydrated in graded ethanol and embedded in Epon. Grids containing the sections were coated with Ilford L4 emulsion by the method of Caro et al. [10] as previously described

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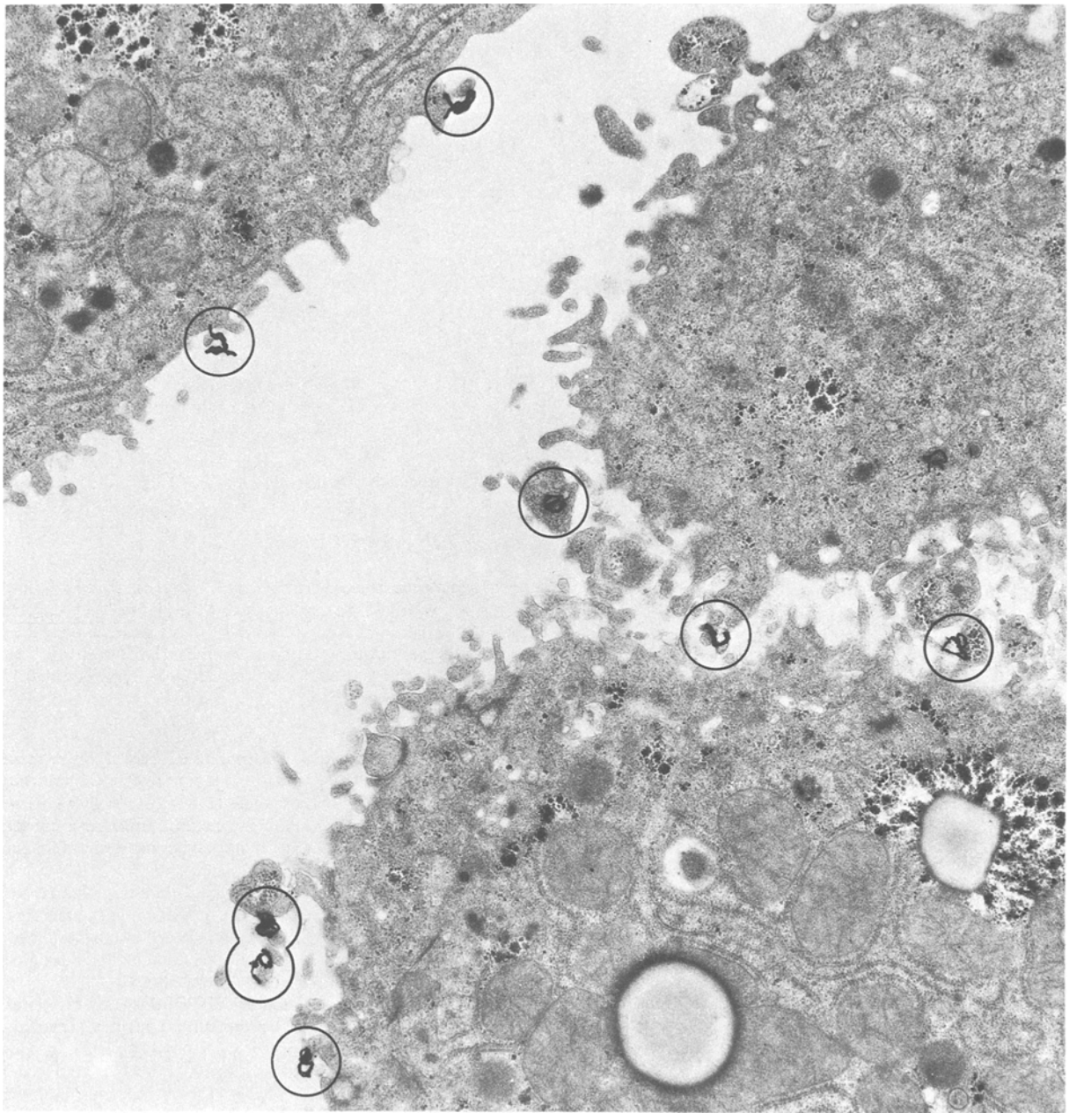

Fig. 1. Thin section of isolated hepatocytes with developed autoradiographic grains (circles). Eighty-five percent of the cells isolated are judged to be morphologically intact as previously described [4]. Examples of cells photographed for developed autoradiographic grains are shown here and it can be seen that these cells are morphologically intact at the E. M. level. These cells have a mean diameter of 22 ± 0.1 μm as measured in the standard incubation medium. Evidence for the functional integrity of these cells is demonstrated by their ability to bind labelled insulin and glucagon and to respond metabolically to catecholamines, glucagon, corticosteroids and insulin (see [4]). This picture represents cells incubated for 5 minutes at 20 °C. × 13,000

[11]. After 3 to 5 weeks, the grids were developed in Microdol X. Samples were then examined in a Philips EM 300 electron microscope (Philips Instruments, Eindhoven, The Netherlands) and grains photographed on all cells that were judged to be well preserved. Photographs were taken at a magnification (9,000 ×) calibrated with a reference grid 2160 lines/mm (Fullam Inc., Schenectady, N. Y.).

Sampling and Analysis of Data

The method of sampling and the analysis of data are similar to those described previously [4, 11]. Only minor points were modified: i a. measurements of the distance between the grain centre and the plasma membrane were all carried out on negatives projected on a Table Projector unit at a final magnification of 47,000 X.

Results

Quantitative Autoradiographic Analysis

When hepatocytes are incubated with ^{125}I-insulin and processed for autoradiography, developed grains are easily identified (Fig. 1). At an appropriate magnification it is possible to measure the distance from the grain centre to the plasma membrane (Fig. 1). As in our previous study in the cultured human lymphocytes, a large number of grains (more than 6000) were photographed avoiding grains related to damaged cells. When measurements of the grain centre to the plasma membrane are made, the data can be expressed in the form of a grain distribution histogram [12, 13]. Using this form of analysis we have shown that in the hepatocyte at 5 min of incubation at 20 °C, the grain distribution is consistant with the initial binding step of insulin to its plasma membrane receptor [2]. If 5 min of incubation at 20 °C is taken as initial localization of ^{125}I-insulin to the plasma membrane, it can be seen that there is a systematic shift in grain distribution as a function of time up to about 60 min when the effect appears to plateau (Fig. 2). A similar relationship has been shown for 37 °C incubations [1, 2].

Background irradiation and non-specific binding (cell associated radioactivity in the presence of a large excess of unlabelled insulin) may influence the shape of the histogram to a small extent [11] and no corrections have been made for these parameters. Background is very low and is only a problem when very few grains are present (see Tables 1 and 2 for the number of grains analyzed for each experiment). For most experiments non-specific binding was 10% or less of total binding (Tables 1 and 2); in addition, the labelled material associated with the cell was primarily intact insulin [2, 4].

Relationship of Binding to Intracellular Translocation

Since translocation occurs at both 20 ° and 37 ° in the hepatocyte (Fig. 2 and Tables 1 and 2), the relative rate of binding could be compared to the translocation process over a broad range of values. At 20 °C ^{125}I-insulin binding approached maximum by 60 min and binding was maintained at roughly a steady state for up to 2 hours (Fig. 3) (Table 1). Translocation of grains began at the first time point measured and maintained a relatively constant relationship to the binding curve. At 37 °C binding was more rapid reaching a maximum by 10–20minutes and falling off slightly after 30 minutes (Fig. 3, Table 2). The translocation process, again, maintained a constant relationship to the binding curve (Fig. 3). When the percent maximal binding was plotted as a percent maximal translocation for all experiments at both 20 ° and 37 °C, (Tables 1 and 2) there was a highly significant positive correlation (r = 0.80) (Fig. 4).

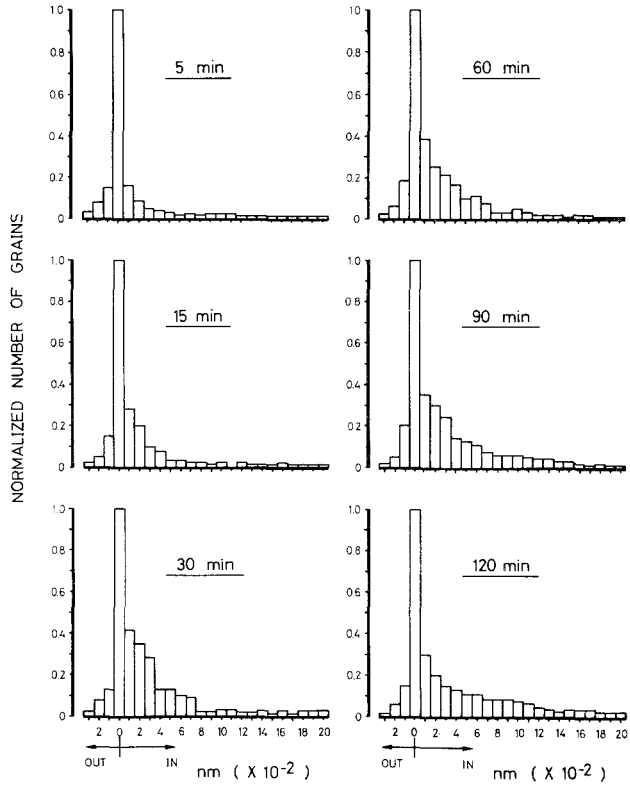

Fig. 2. Distribution of ^{125}I-insulin around the plasma membrane of isolated hepatocytes. For these experiments 1×10^6 cells/ml were incubated at 20 °C for different periods of time shown in Table 1, with 5×10^{-10} mol/l ^{125}I-insulin in 0.5 ml Krebs Ringer bicarbonate buffer, pH 7.7. The normalized number of grains (vertical axis) is plotted as a function of the distance from the grain centre to the plasma membrane (horizontal axis). For number of grains analyzed, see Exp. 3, Table 1

Discussion

The major conclusion of this study is that the binding and internalization of insulin have a high degree of correlation in morphologically and functionally intact isolated hepatocytes.

It is important to note, however, that the term "binding" (HR) has been used synonymously with total cell associated radioactivity. It is apparent that total cell associated radioactivity = HR + internalized ligand when defined in morphologic terms. Thus, we are correlating HR + internalized ligand (vertical axis of Fig. 4) with internalized ligand (horizontal axis of Fig. 4). We have attempted to better

Table 1. Characteristics of cell associated radioactivity from hepatocytes incubated at 20 °C

Time (min)		5	15	30	60	90	120
EXP. # 1	% translocated[a]	–	16.3	–	27.2	40.8	–
	Total number of grains analysed	160	234	–	201	246	–
	% of total ^{125}I-ins.[b] bound	1.10	1.43	1.67	1.72	1.92	2.05
	% of total ^{125}I-ins. non-spec. bound	0.05	0.08	0.11	0.13	0.18	0.20
EXP. # 2	% translocated[a]	–	28.2	–	30.0	–	37.5
	Total number of grains analysed	–	156	–	213	–	195
	% of total ^{125}I-ins.[b] bound	0.56	0.72	0.77	0.87	0.90	0.92
	% of total ^{125}I-ins. non-spec. bound	0.04	0.04	0.06	0.08	0.10	0.10
EXP. # 3	% translocated[a]	0	2.4	17.0	15.3	17.8	21.7
	Total number of grains analysed	180	279	250	251	323	268
	% of total ^{125}I-ins.[b] bound	1.73	3.58	5.46	7.62	8.46	8.21
	% of total ^{125}I-ins. non-spec. bound	0.04	0.08	0.15	0.15	0.15	0.09

[a] To calculate % translocation, the 5 minute time point was used as control. At this time, 77% of grains were contained within ± 250 nm of the plasma membrane; therefore 23% of grains were located beyond 250 nm. For each time point the % translocation is therefore the percent grains beyond 250 nm minus 23%. Percent translocation = grains beyond 250 nm/total grains − (0.23 × 100)
[b] Approximate counts per minute (CPM) bound for each time point can be calculated for both total and non-specific by taking 2×10^5 CPM as tracer concentration of ^{125}I-insulin

Table 2. Characteristics of cell associated radioactivity from hepatocytes incubated at 37 °C

Time (min)		2	5	10	20	30	60
EXP. # 4	% translocated[a]	25.2	37.1	32.1	43.6	34.1	36.2
	Total number of grains analysed	224	201	207	210	213	233
	% of total ^{125}I-ins.[b] bound	0.44	0.71	0.74	0.96	1.11	0.98
	% of total ^{125}I-ins. non-spec. bound	0.06	0.15	0.19	0.24	0.31	0.38
EXP. # 5	% translocated[a]	4.3	13.5	26.5	35.1	38.2	42.8
	Total number of grains analysed	187	192	212	234	219	205
	% of total ^{125}I-ins.[b] bound	1.36	2.57	3.64	4.00	4.06	3.08
	% of total ^{125}I-ins. non-spec. bound	0.04	0.12	0.19	0.28	0.21	0.35

[a] For the method of calculation see Table 1
[b] Approximate CPM bound for each time point can be calculated for both total and non-specific binding by taking 2×10^5 CPM for ^{125}I-insulin

approximate HR and still have a reasonable correlation with internalization (see legend to Fig. 4). The phenomenom described here is a potential factor in all studies in which a labelled ligand is incubated with a cell but has not been generally taken into account.

The study is deficient in several respects; we have been unable to define quantitatively the mechanism of internalization but by analogy to other macromolecules taken up by cells, we assume that the process is one of adsorptive endocytosis. Support for this concept comes from the demonstration that internalized labelled insulin preferentially associates with lysosome-like structures within the cell [3, 4]. Thus, endocytosed membrane would be expected to form endocytotic vesicles which would ultimately associate with lysosomes.

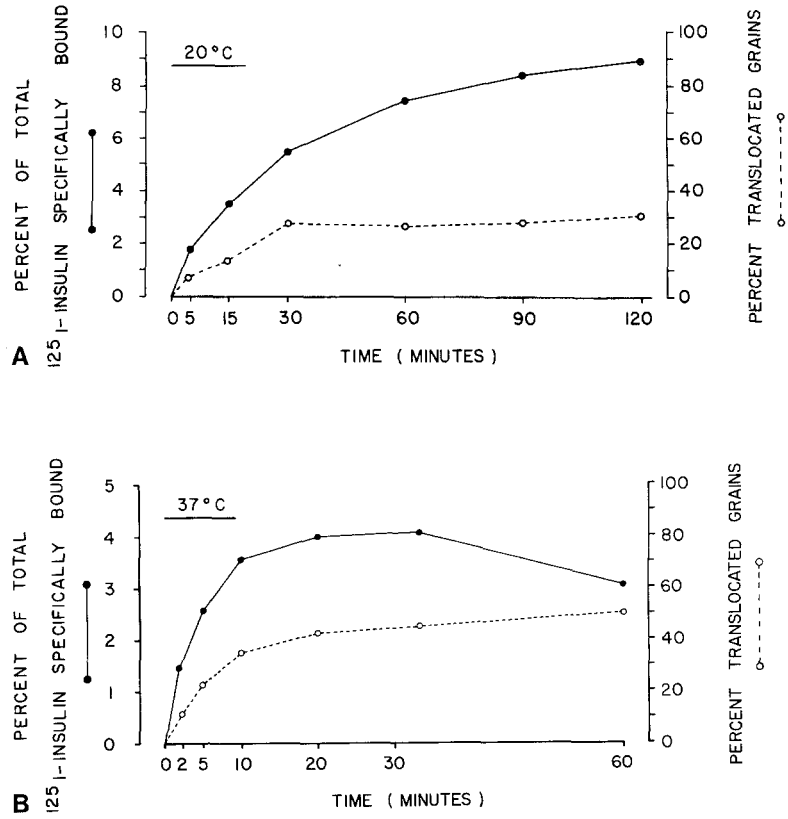

Fig. 3 A and B. Relationship of ^{125}I-insulin binding to translocation of autoradiographic grains at 20 °C **A** and 37 °C **B**. The percent ^{125}I-insulin bound (vertical axis, left ●——●) is plotted as a function of incubation time. The percent translocation (vertical axis right ○---○) is plotted as a function of incubation time. Note that the percent translocation is calculated using the 2 minutes time point at 15 °C of the binding of ^{125}I-insulin to cultured lymphocytes (see [11], Fig. 4) as reference. This is done for illustrative purposes since binding to cultured lymphocytes at 2 min 15 °C probably most closely simulates the initial binding step (compare Figs. 4, 5, 6 in [11]). This mode of expression involves a constant difference throughout the time course of 8% when compared to initial binding to hepatocytes at 5 minutes of incubation at 20 °C (compare with data of Tables 1 and 2 where the translocation is expressed in terms of initial binding of ^{125}I-insulin to isolated hepatocytes incubated for 5 minutes at 20 °C)

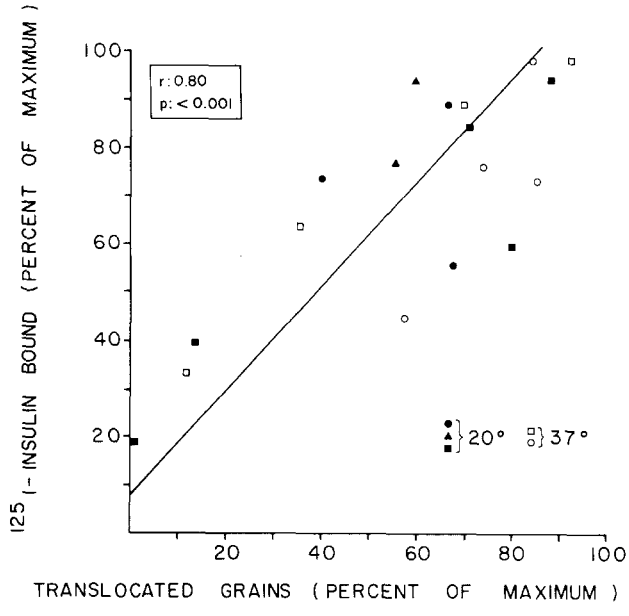

Fig. 4. Relationship of ^{125}I-insulin binding to translocation of autoradiographic grains at 20 °C and 37 °C. All experiments from Tables 1 and 2 are included. ^{125}I-insulin bound calculated as a percent of maximum (vertical axis) is plotted as a function of the translocated grains expressed as a percent of maximum (horizontal axis). The vertical axis is derived from the total cell associated radioactivity which = binding (HR) + internalization. In an attempt to better approximate HR we have carried out the following modification: [total cell associated insulin (pg/ml) – internalized insulin (pg/ml)] was plotted as a function of internalized insulin (pg/ml); under these circumstances r = 0.72

If internalization occurs by way of adsorptive endocytosis then several steps in this process could be involved: these include invagination and pinching off of the invagination to form an endocytotic vesicle [14]. The endocytotic vesicle en route to lysosomes may be further processed by one or more steps and these individual steps must have their respective rate constants.

The demonstration that binding and internalization correlate does not necessarily mean that one event controls the other, but at our present state of information it seems most likely that the binding of

the ligand per se is at least one of the rate controlling steps of internalization. Another example which may be relevant is the binding of ^{125}I-low density lipoprotein (^{125}I-LDL) to its receptors on human fibroblasts. Internalization of ^{125}I-LDL is directly correlated with the binding of the ligand and when intracellular processing is blocked by lysosomal stabilizing agents or in mutant cells the uptake process continues uninterrupted [15]. A similar process appears to apply to epidermal growth factor binding and internalization in human fibroblasts [16]. Further, we have preliminary evidence in isolated rat hepatocytes that when lysosomal stabilizing agents such as ammonium chloride are added to the incubation medium there is a block in intracellular processing without any significant effect on internalization [17].

It is also possible to have binding without internalization in mutant cells [18, 19], in the presence of various chemical agents [20], or by temperature manipulation. Similarly fluid phase endocytosis of agents such as horseradish peroxidase are internalized without binding [14]. These processes, however, are not relevant to normal cells with specific receptors that are incubated under physiologic conditions.

In summary, the data presented are consistent with the view that labelled insulin is internalized in isolated rat hepatocytes by way of ligand-induced endocytosis and that under normal conditions the binding of the ligand is one of the important rate limiting determinants of the internalization process.

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