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CHARACTERISTICS OF INSULIN RECEPTOR BINDING TO VARIOUS RAT TISSUES

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The first step in insulin action is its specific binding to alpha-subunits of the receptor in the plasma membrane. Rats of Wistar strain are commonly used as laboratory animals but there are no data comparing insulin binding by various rat tissues. We studied the insulin binding kinetics in plasma membranes isolated from hearts, livers, brains, skeletal muscles, adipose tissue, thymus and testes in order to compare their ability to bind ^{125}I-insulin and to test which membrane preparation is most useful and convenient for such a study. The dissociation constant (K_d) and the quantity of receptors measured as a binding capacity were determined by the Scatchard method using the LIGAND computer program whereas the binding potency of insulin was calculated as IC_{50} using the ALLFIT computer program. We also introduced the product of $K_a \times R$ (affinity constant multiplied by binding capacity) as an index which describes the functional features of insulin receptors taking into account both number of insulin receptors and their affinity. Taking all the parameters of insulin binding tested together we can conclude that the liver and, to some extent, adipose tissue may provide a useful model for studying hormone — receptor interaction. By contrast, to the group of rat tissues responding rather poorly to insulin belong thymus and testis.

Key words: *insulin receptor, rat tissues.*

INTRODUCTION

Receptors play a fundamental role in the control of cellular biochemical pathways and it is generally accepted that initial step of polypeptyde hormones action is to bind reversibly to stereoscopic receptors on the cell plasma membranes (1, 2). Target organs can modify effect of hormones by changing quantity of receptors molecules and/or their affinity for hormones (3). The first event in insulin action is binding to specific plasma membrane receptor which is a disulphide-linked heterotetrameric transmembrane glycoprotein of about 450 kDa. It consists of two alpha (M_r 135,000) and two beta (M_r 95,000) subunits (4, 5). The alpha-subunits are entirely extracellular and participate in insulin binding, as the N-terminal of the alpha-subunit contains the insulin-binding domain (6, 7, 8). The beta-subunits are composed of three parts: an extracellular domain, a membrane-spanning domain and an intracellular

domain which displays tyrosine kinase activity. Insulin binding results in a conformational change in the receptor molecule which leads to a change in the alpha-alpha subunit interaction within the receptor complex and which is also transmitted to the beta-subunit (9). The tyrosine kinase activity of the insulin receptor appears to be essential for signal transduction, triggering the cascade of biochemical events that lead to the biological effects caused by insulin.

The binding sites discriminate the insulin molecule in a highly selective manner. The biologic specifity of this binding, i.e. strict dependence on the biologically active structure of the insulin molecule, has been well documented (10, 11, 12). Binding defects are manifested by decreased quantity of receptor molecules or decreased affinity of the receptor for insulin. Postreceptor defects may involve transmembrane signalling mechanism or intracellular steps distal to the receptor.

As the first step in insulin action is its specific binding to alpha-subunits we, therefore, studied the insulin binding kinetics by plasma membranes of different rat tissues in order to compare their ability to bind insulin and to test which membrane preparation is most useful and convenient for such a study.

MATERIAL AND METHODS

Male Wistar rats (body weight 200—220 g) were housed in a temperature — controlled room at 20°C and maintained on a standard laboratory rat chow (LSM) with free access to food and water. All experiments were carried out between 9—11 a.m. throughout a whole year. The rats were sacrificed by decapitation and after complete exsanguination hearts, livers, brains, skeletal muscles, adipose tissue, thymus and testes were frozen in liquid nitrogen and stored at −24°C till taken for further analysis.

Isolation of crude membrane fraction

The plasma membranes were prepared according to the method of Havrankowa *et al.* (13). The tissues were homogenized in 20 ml of 0.001 M $NaHCO_3$ and centrifuged at 600 × g for 30 min. The resultant supernatant was centrifuged for 30 min. at 20 000 × g. The membrane preparations were washed twice with 0.001 M $NaHCO_3$. The final pellets were resuspended in 0.04 M Tris — HCl buffer (ph 7.4) containing 0.1% bovine serum albumin (BSA). All the procedures mentioned above were carried out at 4°C. All the chemical reagents were produced by Sigma Chemicals Co., USA.

Binding assay

Insulin — binding activity was measured by incubating the membrane preparations (0.25 mg of protein) at 4°C for 16 h with 80 pg ^{125}I-labelled porcine insulin (specific activity 8.0 Gbq/mg, IBJ Świerk) in a final volume of 0.5 ml 0.04 M Tris buffer (pH 7.4) containing 0.1% BSA.

For the competition binding assay, increasing amounts of unlabelled insulin (Novo, Denmark) were added to the reaction mixture to give a final concentration of 0—700 nmol/l.

Nonspecific binding was determined in the presence of 10 μmol/l unlabelled insulin. Bound and free fractions of insulin were separated by centrifugation at 20 000 × g for 8 minutes and then the radioactivity of the pellets was determined by means of a gamma-counter. The dissociation constant (K_d) and the number of receptors measured as a binding capacity were determined by the

Scatchard method using the LIGAND — PC v. 3.1. computer program (14). The binding potency of insulin was calculated as IC_{50} using the ALLFIT — PC v.2.7. computer program (15).

Student's t-test was used for statistical comparison and differences were considered to be significant if $p<0.05$.

RESULTS

The data presented here confirm that insulin binds to the plasma membranes of different rat tissues including liver, adipose tissue, heart, skeletal muscles, brain, thymus and testes. The highest maximum specific binding was observed in both liver and adipose tissue (*Table 1*). There was no difference between the maximum insulin specific binding (B_{sp}%) in plasma membranes from cardiac muscle and skeletal muscle. The lowest value of B_{sp} was found in plasma membranes isolated from the thymus.

Table 1. Characteristics of insulin receptors in individual rat tissues.

	B_{sp} (%)	HAIRs (10^{-15} mol/mg)	LAIRs (10^{-12} mol/mg	K_{d1} (10^{-9} mol/l	K_{d2} (10^{-9} mol/l	$K_{a1} \times R_1$	$K_{a2} \times R_2$	IC_{50} (10^{-9} mol/l
Liver (1)	11.56±0.90 2,3,4,5,6,7	21.10±1.70 3,6,7	4.53±0.32 3,6,7	0.132±0.026 4,5	151.2±26.9 3,6	0.084	0.015	0.143±0.080 3,4,5,6,7
Adipose tissue (2)	7.42±0.73 1,3,4,5,6,7	39.50±12.60 3,6,7	9.83±6.82	0.270±0.084 7	109.3±42.6 3,4,6	0.073	0.045	0.395±0.110 3,4,6
Heart (3)	3.78±0.39 1,2,7	6.20±1.50 1,2,4,5,7	9.37±1.86 1,5,6,7	0.127±0.023 4,5,6	313.4±62.5 1,2,5,6	0.024	0.015	1.325±0.365 1,2
Skeletal muscle (4)	3.96±0.35 1,2,7	16.80±4.50 3,7	7.58±2.03 6,7	1.091±0.469 1,3,7	349.3±102.6 2,6	0.008	0.011	7.535±3.174 1,2,5
Brain (5)	4.34±0.45 1,2,7	18.70±1.20 3,6,7	4.54±0.47 3,6,7	0.312±0.024 1,3,7	153.1±35.2 3,6	0.030	0.015	0.634±0.110 1,4,6
Testes (6)	3.76±0.26 1,2,7	7.87±3.24 1,2,5	0.91±0.35 1,3,4,5	0.298±0.077 3,7	7.23±0.62 1,2,3,4,5	0.013	0.063	1.900±0.350 1,2,5
Thymus (7)	0.93±0.08 1,2,3,4,5,6	1.34±0.19 1,2,3,4,5	1.31±0.86 1;3,4,5	0.088±0.010 2,4,5,6	142.7±96.7	0.008	0.005	2.219±0.894 1

Data are expressed as means ± SEM for 12 animals

B_{sp} (%) — maximum specific binding of [^{125}I]-insulin to membrane protein
HAIRs — binding capacity of high affinity insulin receptors
LAIRs — binding capacity of low affinity receptors
K_{d1}, K_{d2} — dissociation constans for HAIRs and LAIRs, respectively
$K_{a1} \times R_1$ — association constant for HAIRs multiplied by binding capacity of HAIRs
$K_{a2} \times R_2$ — association constant for LAIRs multiplied by binding capacity of LAIRs
IC_{50} — concentration of unlabelled insulin which reduces maximum specific binding by 50%

Numbers under means indicate differences statistically significant to appropriate tissues ($p<0.05$)

The efffect of increasing concentrations of unlabelled insulin on ^{125}I-insulin displacement in plasma membranes from all tissues tested is shown in *Fig. 1*. Half-maximum displacement of tracer insulin ranged at insulin concentrations from 0.143×10^{-9} mol/l for liver to 7.535×10^{-9} mol/l for skeletal muscle (*Table 1*).

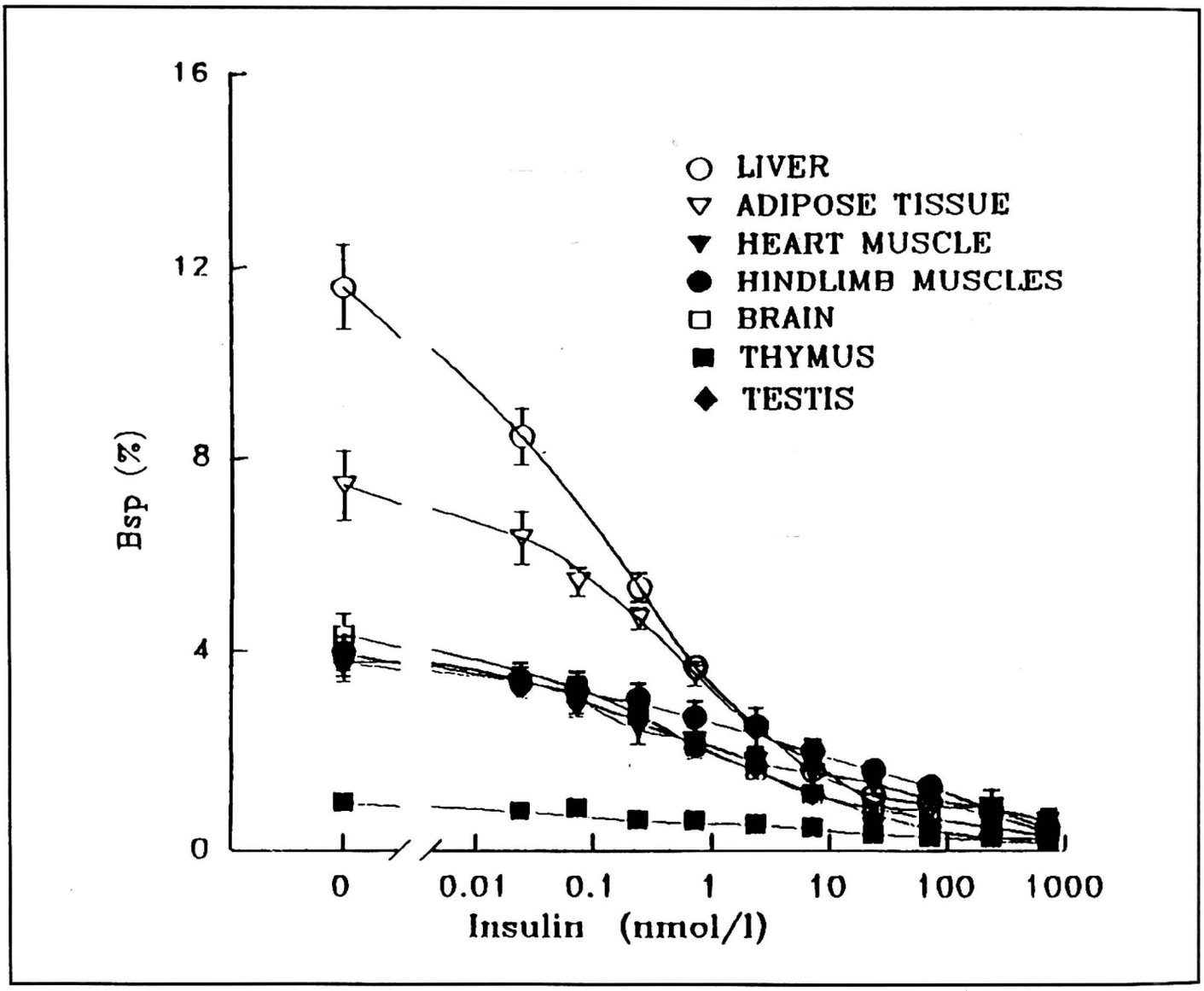

Fig. 1. Displacement of [^{125}I]-insulin by native insuline from the plasma membranes isolated from individual rat tissues. The membranes (0.5 mg protein/ml) were incubated with 80 pg/ml of [^{125}I]-iodoinsulin in the absence or presence of unlabelled insulin over a range of insulin concentration from 0—700 nmol/l. Data are expressed as means ± SEM for 12 animals.

The data of the insulin binding kinetics are presented in the form of a Scatchard plot showing significant differences in the ^{125}I-insulin binding to plasma membranes from the tested tissues (*Fig. 2*). The characteristic features of insulin receptors (dissociation constant, binding capacity and IC_{50}) are summarized in *Table 1*. Analysis of the Scatchard plots indicates that the specific binding fits a model in which two orders of binding sites can be distinguished, with respect to the dissociation constant. This is especially true

for liver and adipose tissue. The only exception is the thymus where the curve exhibits an almost linear shape as compared to the other tissues. This may be explained by the very low value of insulin specific binding in thymus plasma membranes.

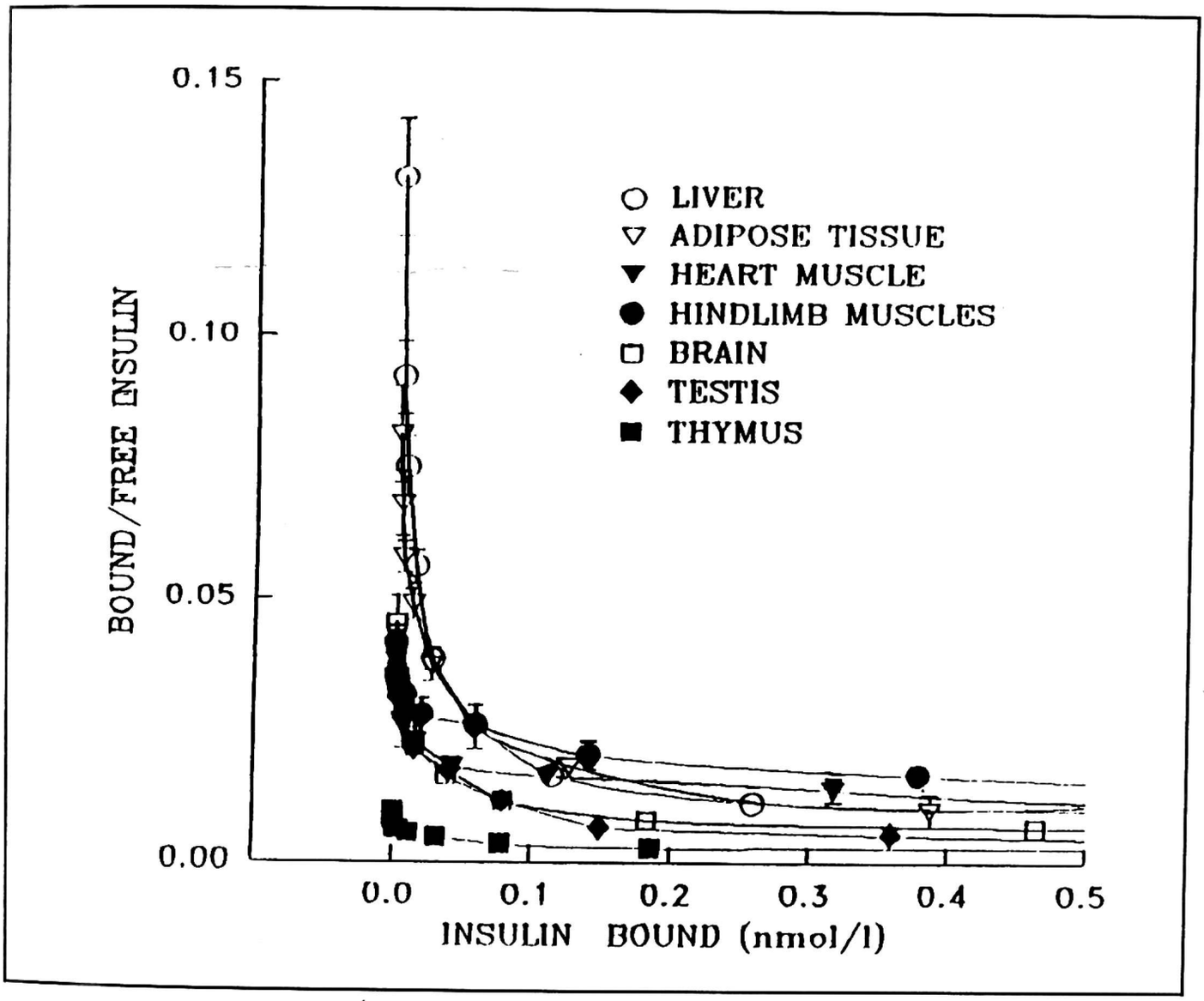

Fig. 2. Scatchard analysis of [^{125}I]-iodoinsulin binding to the plasma membranes obtained from individual rat tissues (bound — free hormone versus hormone bound plotted). Date are expressed as means ± SEM for 12 animals.

As depicted in *Table 1*, the insulin binding sites in skeletal muscle have the highest Kd_1 and Kd_2 values showing that skeletal muscle exhibits the lowest affinity to insulin. The average values of Kd_1 and Kd_2 were found in adipose tissue. The differences between the values of dissociation constants (Kd_1 versus Kd_2) for high affinity (HAIRs) and low affinity (LAIRs) insulin binding sites suggest that the receptor population in every tissue tested is heterogenous with the affinity for hormone. However, an alternative possibility is negative cooperativity between the receptor sites.

Our study demonstrates that the quantity of high affinity insulin receptors, expressed as binding capacity, is higher in adipose tissue than in liver. An opposite situation exists in the case of brain and skeletal muscle, where the quantity of insulin receptors does not differ significantly. The lowest value of insulin receptor quantity was found in thymus plasma membranes which correlates well with the lowest specific binding of insulin in that tissue (the maximum specific insulin binding amounts to only 0.93%). On the other hand, the number of low affinity insulin receptors is apparently highest in plasma membranes from adipose tissue as compared to other tissues including the liver, heart, skeletal muscle, brain, and thymus.

We also found (*Table 1*) that the product of $Ka \times R$ (affinity constant multiplied by binding capacity) for high affinity insulin binding sites (HAIRs) was highest for liver when compared to other tissues. The lowest product was found for plasma membrane preparation from thymus. The product of association constant (Ka) and binding capacity (R) describes the functional features of insulin receptors well (14) indicating their entire ability to bind insulin in any tissue and/or under any conditions. This parameter takes into account both quantity of insulin receptors and their affinity.

DISCUSSION

Insulin receptors have been demonstrated in the cells of a large variety of tissues from different animal species. The values of Kd range from 5×10^{-10} to 5×10^{-8} mol $\times$ liter^{-1} (16). Our present study focused on the characteristics of insulin binding in the different rat tissues commonly used in experiments on insulin receptors. The kinetics of insulin binding to its receptors have been extensively described in the literature published over the past 20 years. However, there is no study on insulin binding being carried out under indentical conditions on so many different rat tissues, at the same time, thereby making the results highly comparable with each other.

Insulin receptors show significant differences as regards their binding kinetics. This is especially true for liver and fat tissue, where the maximal insulin effect is obtained by receptor occupancies of nearly 90% in liver cells compared with only 10% in fat cells (17, 18). This is because binding and metabolic effects show a linear relationship in liver cells whereas in fat cells there is a spare receptor phenomenon.

In isolated rat adipocytes the dose response curve for insulin action is shifted to the left compared with the occupancy of total binding sites or even of high-affinity binding sites (19). Therefore the insulin concentration required to stimulate the adipocyte half-maximally is lower than the dissociation constant for high-affinity binding. Based on the spare receptor concept, it was proposed

that insulin-sensitivity in the isolated adipocyte can be modified either by modification of affinity of binding sites or through changes in insulin receptor number, whereas insulin-responsiveness is due to changes at a postbinding level (20). The pattern found in skeletal muscle clearly differs from the one described in the adipocytes. The results of Camps *et al.* (21) indicate that: 1. maximal insulin action is obtained in skeletal muscle at insulin concentrations which allow full occupancy of high-affinity insulin receptors, 2. full receptor tyrosine kinase activation requires binding to all high-affinity binding sites, 3. there are no spare high-affinity insulin receptors in skeletal muscle. In consequence, the modification of insulin receptor affinity or number in skeletal muscle may have a different impact on insulin action in muscle. For example, the decreased quantity of insulin receptors may result in a diminished maximal response to insulin rather than in a lowered insulin sensitivity. Furthermore, insulin sensitivity in skeletal muscle may be changed in skeletal muscle due to modification of insulin binding, alterations in the coupling between insulin receptor occupancy and tyrosine kinase activation, or in the coupling of other processes occurring at a post-receptor level (22).

According to Azhar and co-workers (23) who examined insulin binding to solubilized insulin from four rat skeletal muscles (*tensor fascia latae, soleus, vastus intermedius* and *plantarius*) no significant differences were noted in the affinity of insulin for its receptor from various muscle types. The values of B_{max} ranged from 4.14—5.54×10^{-12} mol/mg protein whereas Kd values were between 0.280—0.308×10^{-9} mol/l. Our study which was performed on the crude plasma membranes isolated from hind limb skeletal muscles has shown lower values for both the Kd and B_{max} of high-affinity binding sites than reported by Azhar. The reason for this difference could be related to different methods of preparation, usage of solubilized, partially purified receptors, differences in buffer composition or the type of tracer insulin. On the other hand, the Kd values for high affinity insulin receptors ($1.09 \pm 0.47 \times 10^{-9}$ mol/l) found in our experiments are in the range 0.26—2.06×10^{-9} mol/l found by Koeker *et al*, for different skeletal muscles (24). As shown by Suarez *et al.* (25), the binding potency, calculated as the concentration of unlabelled insulin needed to produce a 50% decrase in insulin binding (IC_{50}) in skeletal muscle of normal rats equals $1.9 \pm 0.10 \times 10^{-9}$ mol/l, which is comparable with our results for both cardiac and skeletal muscle.

Rat liver membranes tested in the binding assay exhibited a B_{max} of 1.4 to 1.6×10^{-12} moles per mg protein (16). As described by Kahn and his co-workers (26) liver plasma membranes have shown a heterogenous population of receptor sites. The data fit a model with two main classes of insulin receptor sites: a high affinity-low capacity site with Ka of 2.0×10^{9} M^{-1} and

capacity of 5×10^{-14} moles per mg of membrane protein and a low affinity-high capacity site with Ka of 2.1×10^{8} M^{-1} and capacity of 15×10^{-12} moles per mg of membrane protein. In our present study we have also shown the presence of high affinity sites (B_{max} 22.1×10^{-15} mol/mg, Kd 0.132×10^{-9} mol/l and low affinity sites (B_{max} 4.53×10^{-12} mol/mg, Kd 151.2×10^{-9} mol/l).

The properties of insulin receptors in the heart are, in many respects, similar to those described for the insulin receptors in the liver (27). These similarities include some kinetic parameters, such as the apparent heterogeneity of the binding sites with respect to equilibrium constants and the time temperature dependence of the binding , with a slower, but higher, binding of insulin at lower temperatures (28).

Insulin binding to rat myocardial membranes (29) showed a binding capacity of $5.5 \pm 4.8 \times 10^{-12}$ mol/g protein, Ka of $5.2 \pm 4.1 \times 10^{9}$ (M^{-1}) and maximum specific insulin binding of $3.03 \pm 1.67\%$ for high affinity insulin receptors. These results are comparable with ours. Insulin receptors in the central nervous system conform to the characteristics described for receptors in peripheral tissues. The one exception noted is the lower molecular weights of the alpha and beta subunits of receptors from the brain (30). In our experiments the data obtained for brain insulin receptors resembles, in many respects those for cardiac and skeletal muscles and testes as well.

As shown by Kimura and co-workers (31) Leydig cells were shown to posses insulin-binding sites on their plasma membranes. Our study, which was performed on crude plasma membranes, confirms these data showing the curvilinearity of binding and giving some more details on receptor kinetics.

It has been known that all thymic epithelial cells and some lymphoid ones bind insulin during fetal life. (32). According to the concept generally accepted, mature non-stimulated T-lymphocytes do not bind insulin which may be an explanation of our results indicating weak insulin binding in the thymus.

Insulin binding displays complex kinetics characterized by curvilinear Scatchard plots. The existence of negative cooperativity for insulin has been proposed as an explanation for this complexity (27).

Our conclusions drawn from an *in vitro* study, are consistent with the results of Whitcomb and his co-workers (36) who, using a unique *in vivo* radioreceptor assay, found that binding per gram wet weight was greatest in the liver. By contrast, a small but specific insulin binding of 3 pg/g in epididymal fat was observed. However, sensitivity and responsiveness of various metabolic processes to insulin *in vivo* is known to be higher in epididymal adipose tissue than other tissues including the liver (37).

Presented in our paper comparative description gives a wide review of insulin binding sites in many rat tissues and shows both organs very sensitive to insulin, and those responding rather poorly to the hormone. To the second group belong certainly thymus and testis. Simultaneously, to some degree

unexpecting is high insulin binding to brain membranes. Other conclusions drawn from our investigations allow us to state that both liver and to some degree adipose tissue — despite their different physiological role — are under a huge influence of insulin and can be a useful model for studying hormon-receptor interaction in various physiological states of an animal organism.

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