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Evidence for the lack of spare high-affinity insulin receptors in skeletal muscle

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In this study, the relationship between the concentration of extracellular insulin, insulin binding and insulin action was evaluated in skeletal muscle. Initially we investigated the dose–response relationship of insulin action using three different experimental models that are responsive to insulin, i.e. the isolated perfused rat hindquarter, incubated strips of soleus muscle, and insulin receptors partially affinity-purified from skeletal muscle. We selected as insulin-sensitive parameters glucose uptake in the perfused hindquarter, lactate production in the incubated muscle preparation, and tyrosine receptor kinase activity in the purified receptor preparation. Our results showed that the dose–response curves obtained in the perfused hindquarter and in the incubated muscle were superimposable. In contrast, the dose–response curve for insulin-stimulated receptor tyrosine kinase activity in partially purified receptors was displaced to the left compared with the curves obtained in the perfused hindquarter and in the incubated muscle. The differences between the dose–response curve for receptor tyrosine kinase and those for glucose uptake and lactate production were not explained by a substantial insulin concentration gradient between medium and interstitial space. Thus the medium/interstitial insulin concentration ratio, when assayed in the incubated intact muscle at 5°C, was close to 1. We also compared the dose–response curve of insulin-stimulated receptor tyrosine kinase with the pattern of insulin-binding-site occupancy. The curve of insulin-stimulated receptor kinase activity fitted closely with the occupancy of high-affinity binding sites. In summary, assuming that the estimation of the medium/interstitial insulin concentration ratio obtained at 5°C reflects the actual ratio under more physiological conditions, our results suggest that maximal insulin action is obtained in skeletal muscle at insulin concentrations which do allow full occupancy of high-affinity binding sites. Therefore our data provide evidence for a lack of spare high-affinity insulin receptors in skeletal muscle.

INTRODUCTION

Skeletal muscle is a principal target tissue for insulin action and, in fact, insulin-stimulated glucose disposal under euglycaemic clamp conditions largely represents uptake into skeletal muscle [1,2]. Furthermore, the response to insulin in skeletal muscle is known to be subject to regulation, and can be depressed (as reported in diabetes, obesity, denervation or pregnancy [3–10]) or enhanced (as occurs after acute or chronic exercise [11–14]). These modifications in the response to insulin by skeletal muscle influence the rate of overall glucose utilization by the organism.

A complete picture of the biochemical pathway activated by insulin is not available. However, an initial step of insulin action is the interaction of the hormone with membrane-anchored receptors [15,16], which is followed by the triggering of insulin receptor kinase activity (see [17,18] for reviews). A question that still needs to be resolved is the exact nature of the relationship between occupancy of insulin-binding sites and insulin action. This is an extremely complicated issue, due, at least in part, to the fact that insulin binding displays complex kinetics characterized by curvilinear Scatchard plots. The existence of negative co-operativity for insulin binding has been proposed as an explanation for the complex insulin-binding kinetics [19,20], but this model is in conflict with much published data [21–23]. On the other hand, there is evidence that the insulin receptor has a high affinity for only one molecule of insulin [24,25], and so data could also be explained on the basis of receptor populations with different equilibrium constants for the formation of insulin–receptor complexes. A further difficulty arises from studies in isolated adipocytes, in which it has been demonstrated that maximal insulin action is attained before all high-affinity binding sites are occupied with the ligand [26,27] and in which a partial decrease in insulin binding does not modify the maximal reponse to insulin [28]. This suggests the existence of spare receptors. However, whether all insulin-sensitive tissues display spare high-affinity receptors is not clear.

In the present study we have attempted to evaluate the relationship between the occupancy of insulin-binding sites and insulin action in skeletal muscle. To this end, we have investigated the dose–response relationship of insulin action using three different experimental models which are sensitive to insulin; the isolated perfused rat hindquarter, the incubated muscle, and the insulin receptor partially purified from skeletal muscle. Furthermore, we have compared the dose–response curve obtained for the partially purified receptor preparation with the pattern of insulin-binding-site occupancy estimated according to the model of two non-interacting binding sites and with the negative co-operativity model.

MATERIALS AND METHODS

Materials

Porcine monocomponent insulin was a gift from Mr. T. L. Jeatran, Eli Lilly & Co. (Indianapolis, IN, U.S.A.). [^{125}I-Tyr^{A14}]-Monoiodoinsulin was obtained from New England Nuclear. [γ-^{32}P]ATP was prepared from [^{32}P]P_i (New England Nuclear) using a Gamma-prep kit from Promega Biotech. All electrophoresis reagents were obtained from Bio-Rad, wheat-germ agglutinin (WGA) bound to agarose was obtained from Vector,

Abbreviation used: WGA, wheat-germ agglutinin.
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and BSA for perfusion experiments was from Pentex Corporation. Fatty-acid-free BSA (fraction V) and most commonly used chemicals were from Sigma.

Perfusion studies

Male Wistar rats (200–250 g) obtained from our own colony were used. The rats were fed on Purina Laboratory chow *ad libitum*. Animals were housed in animal quarters maintained at 22°C with a 12 h-light/12 h-dark cycle. On the day of the experiment, rats were anaesthesized with an intraperitoneal injection of pentobarbital (5–7 mg/100 g body weight) and their hindquarters were perfused by the method of Ruderman *et al.* [29], as modified by Goodman and co-workers [30]. The perfusion medium contained Krebs–Henseleit solution, aged/rejuvenated human red cells [31] added to obtain a haematocrit of 30%, 4% BSA, 6 mM-glucose, 0.15 mM-pyruvate and 1.5–2.0 mM-L-lactate (originating from the erythrocytes). Porcine insulin was added at concentrations ranging from 0 to 130 nM. The initial volume of perfusate was 150 ml. Immediately before placement in the perfusion apparatus, the rats were killed by an intracardiac injection of pentobarbital.

The hindquarters were initially perfused for 17 min to allow equilibration. The first 25 ml of perfusate that passed through the preparation was discarded, whereupon the medium was recirculated at a flow rate of 12.5 ml/min. After the equilibration period (time 0) and at 10, 20 and 30 min thereafter, samples were taken from the perfusate reservoir and placed in 2 vol. of ice-cold 6% (w/v) $HClO_4$ for glucose assay [30]. Rates of glucose uptake by the hindquarters were determined from changes in its concentration in the perfusate, and are expressed as $\mu mol \cdot h^{-1} \cdot g^{-1}$. Glucose uptake by erythrocytes over 30 min was negligible.

Studies with incubated muscle

The dissection and isolation of strips of soleus muscle from rats was carried out under anaesthesia with pentobarbital (5–7 mg/100 g body wt., intraperitoneally) by a modification of the method of Crettaz *et al.* [6]. Only strips weighing 25 mg or less were used. The strip of soleus muscle was fixed to a stainless steel clip in order to maintain the muscle under slight tension (approximating to the resting length) during the incubation. Such muscles are able to maintain normal ATP (2.5 ± 0.6 μmol/g of tissue and 2.6 ± 0.6 μmol/g of tissue *in vivo* and after 2 h of incubation respectively) and phosphocreatine concentrations (11.6 ± 1.3 μmol/g of tissue and 10.8 ± 1.6 μmol/g of tissue *in vivo* and after 2 h of incubation repsectively) during a 2 h incubation.

Strips of soleus muscle were incubated in a shaking incubator at 37°C for 90 min in 3 ml of Krebs–Henseleit buffer, pH 7.4, containing 5 mM-glucose, 0.2% BSA, 20 mM-Hepes and different concentrations of insulin. After addition of the muscle strips to the vials, the latter were stoppered and placed in a Dubnoff metabolic shaker set at 37°C and a shaking rate of 60 cycles/min. Vials were gassed with O_2/CO_2 (19:1) during the entire incubation period. The incubation medium was kept for 60 min, and then renewed for measurement of lactate production during the last 30 min of incubation [30]. In a separate set of experiments, soleus muscles were incubated in the presence of 10 μM-[^{3}H]inulin (330 μC/mmol) for 30 min. The amount of radioisotope present in the samples was determined, and this information was used to calculate the extracellular space (0.32 ± 0.02 ml/g), as previously reported [12].

Soleus muscles were also incubated in the presence of 1 nM-[^{125}I-Tyr^{A14}]insulin for 2, 3 or 4 h at 5°C, to prevent internalization and degradation of insulin. After the incubation period, muscles were washed five times (5 min/wash) in cold 0.9% NaCl containing 0.25% BSA, or just blotted in filter paper. This wash step (5 washes, 5 min/wash) is enough to eliminate unbound insulin distributed in the extracellular space without decreasing specific binding [4,12,32]. Radioactivity was quantified in a Packard γ-radiation counter. Insulin present in the extracellular space was assessed approximately as the difference between the radioactivity present in muscles not subjected (bound insulin plus insulin in the extracellular space) and subjected (bound insulin) to the washing step after the incubation period. The concentration of insulin in the extracellular space in intact muscles was calculated by taking into account the extracellular space determined with insulin as described above.

Preparation of insulin receptors

Hindlimb muscle was obtained from pentobarbital-anaesthesized rats as previously described [33,34]. In brief, muscles (3–4 g) were frozen in liquid nitrogen, powdered in a cooled mortar and then homogenized using a Polytron homogenizer (setting 5, 30–40 s, 4°C) in a buffer (2:7, w/v) containing 25 mM-Hepes, 4 mM-EDTA, 4 mM-EGTA, 1 trypsin-inhibitory unit of aprotinin/ml, 2 mM-phenylmethanesulphonyl fluoride, 25 mM-benzamidine, 2 μM-leupeptin, 2 μM-pepstatin, 1 mM-bacitracin, 10 mM-NaF, 0.1 mM-sodium orthovanadate and 10 mM-sodium pyrophosphate, pH 7.4. Following homogenization, Triton X-100 (1%) was added, and the homogenate was stirred for 1 h at 4°C and centrifuged at 150000 ***g*** for 90 min at 4°C. The 150000 ***g*** supernatant (6 ml) was recycled three times through a column containing 2 ml of WGA bound to agarose, at 4°C. The resin was washed with buffer (75 ml) containing 25 mM-Hepes and 0.1% Triton X-100, pH 7.4. Receptors were eluted from the WGA column with buffer containing 25 mM-Hepes, 0.1% Triton X-100 and 0.3 M-*N*-acetyl-D-glucosamine, pH 7.4.

Ligand binding

Insulin binding was measured as in [33]. WGA eluate (20 μl) was incubated in 30 mM-Hepes containing 0.1% BSA and 100 units of bacitracin/ml (pH 7.6, 1 h, 22°C, 200 μl), 20000 c.p.m. [^{125}I-Tyr^{A14}]monoiodoinsulin ($\sim$ 60 pM) and increasing concentrations of unlabelled insulin. Receptors were precipitated with 0.5 ml of bovine γ-globulin (1 mg/ml) and 0.5 ml of poly(ethylene glycol) (25%, w/v). Non-specific binding was estimated as ^{125}I-insulin bound in the presence of 1 μM-insulin (5–10% of total binding). Binding data were expressed per μg of protein, with the latter measured using the method of Bradford [35].

Determination of concentration of hormone–receptor complexes

Ligand binding data were analysed according to two different models, i.e. the negative co-operative binding model [19,20] and the two-site model [22,36].

When binding data were analysed according to the negative co-operative model, fractional occupancy was defined as a ratio between the concentration of bound ligand (B) and the maximal concentration of bound ligand (R_0). K_e, K_f and R_0 were calculated as in [37].

Binding data were also analysed using the curve-fiting program ENZFITTER (Elsevier Biosoft), which provides kinetic analysis based on a two-site model. This provides estimates of dissociation constants and concentration of binding sites for high-affinity and low-affinity receptors (K_{d_1}, K_{d_2}, R_{o_1} and R_{o_2}). According to this model, two different equilibria are considered:

$$H + R_1 \underset{K_{d_1}}{\rightleftharpoons} HR_1$$

and

$$H + R_2 \underset{K_{d_2}}{\rightleftharpoons} HR_2$$

where H is the concentration of free hormone; R_1 is the concentration of free high-affinity binding sites; R_2 is the concentration of free low-affinity binding sites; HR_1 is the concentration of occupied high-affinity binding sites, and HR_2 is the concentration of occupied low-affinity binding sites. Therefore, two different dissociation constants can be defined:

$$K_{d_1} = \frac{H \cdot R_1}{HR_1}$$

and

$$K_{d_2} = \frac{H \cdot R_2}{HR_2}$$

The fractional occupancy of high-affinity (F_{oc_1}) and low-affinity (F_{oc_2}) binding sites can be calculated at different concentrations of total hormone (H_o), as follows:

$$F_{oc_1} = \frac{H}{K_{d_1} + H}$$

and

$$F_{oc_2} = \frac{H}{K_{d_2} + H}$$

where H is a value found experimentally, at a given concentration of total hormone.

Phosphorylation of an exogenous substrate

The receptor preparation was preincubated for 1 h in 30 mM-Hepes buffer, pH 7.6, containing 50 mM-magnesium acetate, 4 mM-$MnCl_2$ and various concentrations of insulin. The receptor kinase activity was initially activated by the addition of 50 μM-[γ-^{32}P]ATP (5–10 μCi) for 10 min. The reaction was initiated by the addition of the exogenous substrate (copolymer of Glu/Tyr, 4:1; 0.25 mg/ml). The reaction was stopped after 30 min by applying samples to filter paper squares (Whatmann 3MM), which were immediately washed in 10% trichloroacetic acid containing 10 mM-sodium pyrophosphate. Papers were washed, dried and counted for radioactivity as described [33]. Non-specific binding of [γ-^{32}P]ATP to the paper accounted for 5–10% of the radioactivity associated with the copolymer in the absence of insulin. All assays were carried out in triplicate.

RESULTS

Dose–response relationship of insulin action

The dose–response curve of insulin-stimulated glucose utilization by the perfused rat hindquarter was initially evaluated. Under our conditions, insulin caused a 4-fold increase in glucose uptake by the isolated perfused rat hindquarter (Fig. 1*a*); a half-maximal effect of insulin was detected at 1.9 nM (Fig. 1*a*).

Next, the effect of insulin on lactate production was investigated in strips of soleus muscle incubated in the presence of 5 mM-glucose. In the incubated muscle, lactate production is an appropriate index of glucose utilization [38], since glucose is mainly directed to lactate synthesis, and less than 10% of glucose is either incorporated into glycogen or oxidized to CO_2. Insulin caused a 3-fold increase in lactate production, and in keeping with data obtained in the perfused hindquarter, a half-maximal effect of the hormone was attained at 1.8 nM (Fig. 1*b*).

Furthermore, an early effect of insulin, i.e. the activation of insulin receptor tyrosine kinase for exogenous substrates, was investigated in detergent-solubilized insulin receptor preparations partially purified from skeletal muscle (Fig. 1*c*). In contrast to studies with the perfused rat hindquarter and the incubated muscle, the tyrosine kinase activity of the insulin receptor was assayed at 25°C. This was because incubation of receptors at 37°C caused a total inactivation of tyrosine kinase activity, regardless of the ionic strength of the medium (results not shown). This is in keeping with previous observations performed in the partially purified rat liver insulin receptor preparation [39]. Therefore analysis of insulin-stimulated tyrosine kinase activity was performed at 25°C and in the absence of NaCl in the medium, conditions under which the insulin-binding affinity (K_d of high-affinity binding sites = 0.4 nM) is similar to the affinity

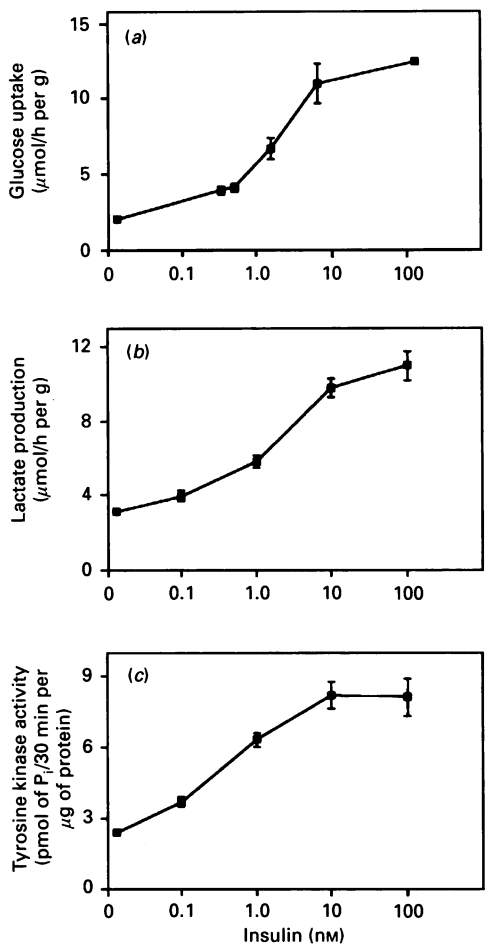

Fig. 1. Biological effects of insulin in muscle and in the partially purified muscle insulin receptor preparation

(*a*) Effects of insulin on glucose uptake by the isolated perfused rat hindquarter. Values are means ± S.E.M. of 6–12 experiments. Insulin at the indicated concentrations was added to the initial cell-free perfusate. Glucose utilization was evaluated from the change in glucose concentration in the perfusate. (*b*) Effects of insulin on lactate production by strips of soleus muscle incubated *in vitro*. Values are means ± S.E.M. of 5–6 observations per group. (*c*) Kinase activity of insulin receptors from skeletal muscle towards an exogenous substrate. Insulin receptors were partially purified from skeletal muscle as described [33,34]. The partially purified receptor preparation (10 μl) was incubated for 30 min at 22°C in 30 mM-Hepes buffer, pH 7.6, containing 50 mM-magnesium acetate, 4 mM-$MgCl_2$ and various concentrations of insulin. [γ-^{32}P]ATP (50 μM) was added and samples were incubated for an additional 10 min. The substrate (copolymer of Gly/Tyr, 4:1; 0.25 mg/ml) was then added and allowed to react for 30 min. Each data point is the mean ± S.E.M. of six observations per group performed in triplicate.

found at 37°C in the presence of 100 mM-NaCl (K_d of high-affinity binding sites = 0.28 nM). Under these conditions, insulin caused a 4-fold increase in receptor kinase activity; the maximal insulin effect was found at 10 nM and the half-maximal effect at 0.5 nM.

When data shown in Fig. 1 were recalculated to give percentages of the maximal effect of insulin (Fig. 2), the curves obtained with the perfused hindquarter and the incubated muscle were found to be superimposable; in contrast, the curve obtained by plotting tyrosine receptor kinase activity data from the partially purified receptor preparation was markedly displaced to the left.

To investigate the reason for these differences, we determined the concentration of insulin in the extracellular space of intact soleus muscle. To this end, muscles were incubated in the presence of 1 nM-^{125}I-insulin at 5°C for different times, after which muscles were either washed five times with cold 0.9% NaCl (5 min/wash; to eliminate unbound ^{125}I-insulin from the interstitial space) or not washed. The estimation of extracellular insulin in the intact soleus muscle was assessed as the difference between results with the two conditions (unwashed and washed muscles). The experiments were done at 5°C rather than at 37°C to prevent insulin internalization and degradation; however, this low temperature can affect the rate of diffusion and the size of the extracellular space in the muscle fibre. In consequence, this procedure provides only a very approximate estimate of the concentration of insulin in the interstitial space in the muscle fibre. Using such an approach, we found that 2 h after the start of the incubation with 1 nM-^{125}I-insulin, insulin had already reached 72% of its final distribution in the interstitial space (concentration of insulin in the interstitial space was 0.63 ± 0.15 nM) (Fig. 3). Furthermore, after 3 h of incubation in the presence of 1 nM-insulin, the concentration of hormone in the interstitial space was 0.88 ± 0.09 nM (Fig. 3), i.e. similar to values obtained after 4 h of incubation. In contrast, insulin binding increased progressively during the whole of this time period (Fig. 3). Under our conditions, the medium/interstitial insulin concentration ratio was 1.1:1, i.e. reasonably close to previous estimates of the plasma/lymph ratio [40]. Assuming a constant medium/interstitial insulin concentration ratio for all medium concentrations investigated, the actual dose–response curve in the intact muscle is not shifted appreciably to the left compared with data shown in Fig. 2. Therefore the differences between dose–response curves obtained *in vivo* and in the partially purified receptor preparation is not a consequence of a limited passage of insulin to the interstitial space in the muscle.

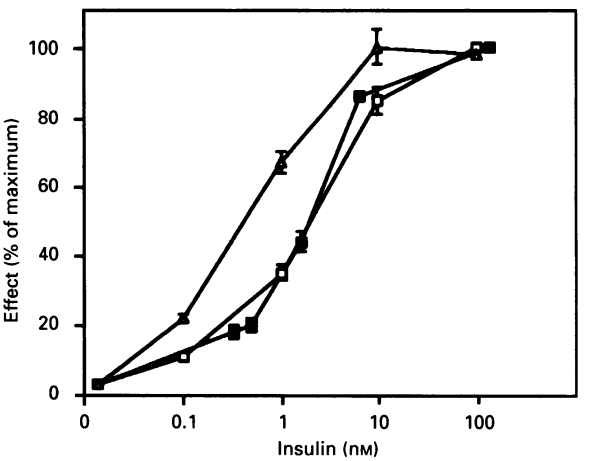

Fig. 2. Insulin effects on glucose uptake by perfused hindquarter (■), lactate production by incubated soleus muscle (□) and tyrosine receptor kinase activity of partially purified insulin receptors (△)

Data correspond to values presented in Fig. 1 expressed as a percentage of the maximal effect of insulin (means ± S.E.M.). These values were determined by dividing the increment due to insulin at each hormone concentration (stimulated value minus basal value) by the maximal increment due to insulin (at approximately 100 nM). For details, see the legend to Fig. 1 and Materials and methods section.

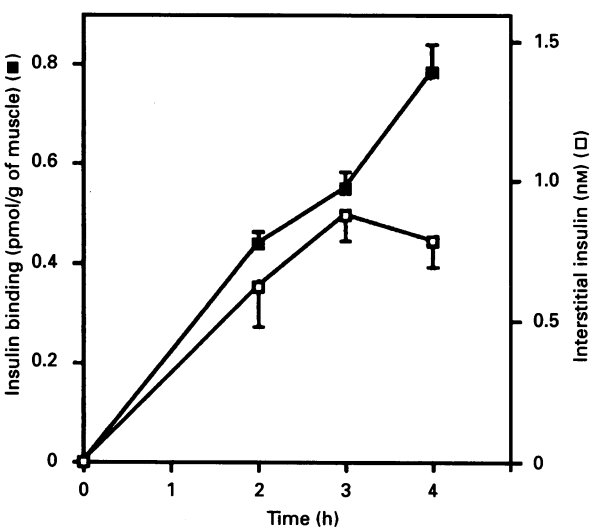

Fig. 3. Concentration of insulin in the interstitial space of the incubated soleus muscle

Values are means ± S.E.M. of 4–7 observations per group. Soleus muscles were incubated at 5°C in the presence of 1 nM-^{125}I-insulin. After 2, 3 and 4 h of incubation, insulin binding (■) and the concentration of insulin in the interstitial space (□) were assessed as described in the Materials and methods section.

Relationship between insulin receptor occupancy and insulin action

In an attempt to relate the dose–reponse curves of insulin action to occupancy of binding sites, insulin binding was assessed using a solubilized insulin receptor preparation partially purified from skeletal muscle. This model was selected because the insulin-binding assay is performed more precisely in the solubilized receptor preparation than in intact muscle preparations. Competition of insulin binding for the solubilized partially purified insulin receptor preparation was analysed by the method of Scatchard (Fig. 4). Insulin binding produced a curvilinear Scatchard plot with a break at 1–3 nM. Insulin binding to the receptor was generally observed to be linear in the insulin concentration range from 0.05 to 1 nM, which is similar to that in other studies carried out over this insulin concentration range after purification of the insulin receptor from skeletal muscle [33]. The insulin-binding parameters were analysed (Table 1) assuming either a non-interacting two-site model [22,36] or a negative co-operativity binding model [19,20,37]. The analysis of parameters according to the two-site binding model indicated that the dissociation constants for high-affinity and low-affinity binding sites differed by a factor of approx. 100-fold. In addition, the number of low-affinity binding sites was 6-fold greater than that of high-affinity binding sites (Table 1).

Subsequently, binding data were recalculated in order to assess the fractional occupancy of high-affinity binding sites, as well as the occupancy of total insulin-binding sites (where binding varies between empty and filled sites according to the negative co-operative binding model), in the presence of different concentra-

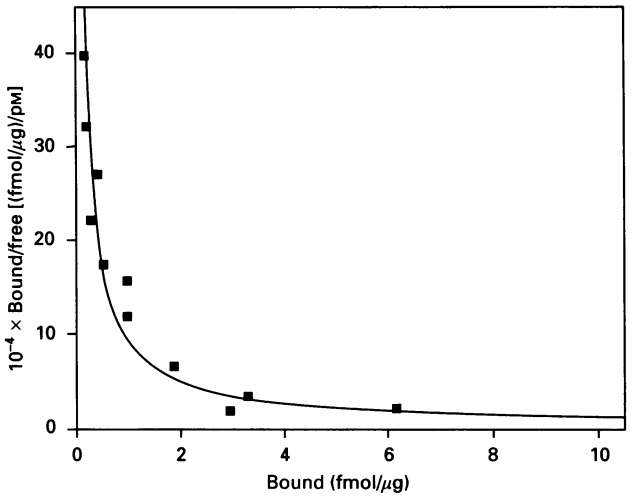

Fig. 4. Insulin binding in skeletal muscle

Insulin receptors were partially purified from skeletal muscle homogenates following solubilization in Triton X-100 and ultracentrifugation using lectin affinity chromatography. The WGA eluate (20 μl) was incubated for 1 h at 22°C in a buffer containing 25 mM-Hepes, 0.1 mg of BSA/ml, 100 units of bacitracin/ml, ^{125}I-insulin, and various concentrations of unlabelled insulin in a total volume of 200 μl. See the Materials and methods section for further details. The data shown are from a representative Scatchard plot, and each point is the mean of triplicate determinations.

Table 1. Insulin-binding parameters analysed by both two-site and negative co-operative binding models for insulin receptor partially purified from skeletal muscle

Results are means ± S.E.M. of eight independently performed experiments. Insulin receptors were partially purified from skeletal muscle homogenates following solubilization in Triton X-100 and ultracentifugation using lectin affinity chromatography. The WGA eluate (20 μl) was incubated for 1 h at 22°C in a buffer containing 25 mM-Hepes, 0.1 mg of BSA/mg, 100 units of bacitracin/ml, ^{125}I-insulin and various concentrations of unlabelled insulin in a total volume of 200 μl. Estimates of parameter values according to the two-site binding model were determined by the curve-fitting ENZFITTER program. Estimates of parameter values according to the negative co-operative binding model were determined as in [37].

Model	Parameter	Value
Two-site binding model	1/Association constant (nM^{-1})	
	$1/K_1$	0.4 ± 0.1
	$1/K_2$	62 ± 22
	Binding site (fmol/μg of protein)	
	R_{o2}	1.1 ± 0.3
	R_{o2}	6.8 ± 2.2
Negative co-operative binding model	1/Association constant (nM^{-1})	
	$1/K_e$	2.0 ± 0.4
	$1/K_p$	13 ± 4
	Binding site (fmol/μg of protein)	
	R_o	8.8 ± 2.1

tions of insulin in the incubation medium. The occupancy of high-affinity binding sites was such that half-maximal occupancy was attained at approx. 0.4 nM-insulin and maximal occupancy was found at 10 nM-insulin (Fig. 5). On the other hand, the occupancy of low-affinity binding sites was negligible at concentrations of 1 nM or less. At 10 nM-insulin, although fractional occupancy of low-affinity binding sites only accounted for 14% of the total, it represented, in absolute terms, the same degree of occupancy as that of high-affinity binding sites (results not shown). Half-maximal occupancy of low-affinity binding sites was attained at a concentration around 60 nM-insulin (result not shown). When occupancy of total binding sites was assessed, half-maximal occupancy was found at approx. 60 nM-insulin and maximal occupancy was only attained at 1 μM-insulin (Fig. 5). This latter is in keeping with previous observations obtained in the incubated muscle [5].

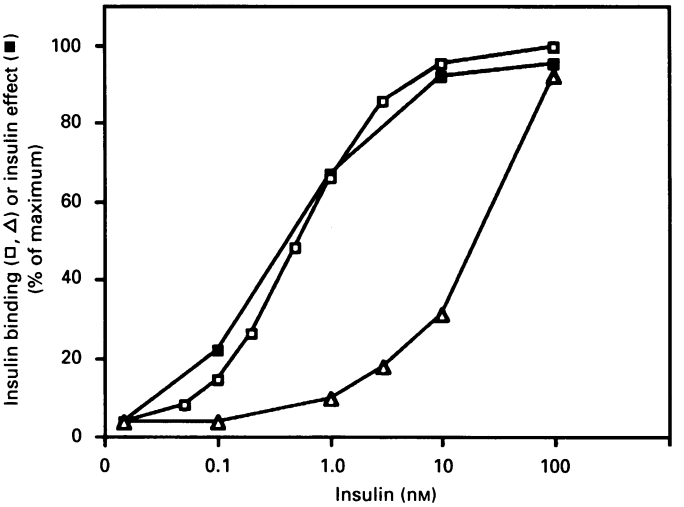

Fig. 5. Relationship between dose–response curves of receptor occupancy and insulin-stimulated tyrosine receptor kinase activity of purified insulin receptors from skeletal muscle

Insulin binding parameters were analysed according to the two-site model or to the negative co-operative binding model. Occupancy of high-affinity binding sites (□) as well as occupancy of total binding sites (affinity varying between empty and filled site, △) was calculated as explained in the Materials and methods section. Data on occupancy of binding sites are expressed as a fractional occupancy and are from a representative Scatchard plot. Insulin-stimulated receptor kinase data (■) correspond to values presented in Fig. 1, expressed as percentages of the maximum effect of insulin. This was determined as explained in the legend to Fig. 2.

Finally, we correlated the fractional occupancy of high-affinity or total binding sites with insulin-stimulated receptor tyrosine kinase activity with exogenous substrates. The stimulatory effect of insulin on receptor kinase activity (as a percentage of the maximum) and fractional occupancy data were compared (Fig. 5). The dose–response curve of the effect of insulin on receptor tyrosine kinase activity was in good agreement with the occupancy of high-affinity binding sites as a function of insulin concentration (Fig. 5). Curves of occupancy of total binding sites as a function of insulin concentration were markedly displaced to the right compared with the dose-response of tyrosine kinase activation (Fig. 5).

DISCUSSION

The results of this study demonstrate a close similarity between the dose–response curves of insulin-stimulated glucose uptake by the perfused rat hindquarter and insulin-activated lactate production by incubated rat soleus muscle. The dose–response curve for insulin-stimulated receptor tyrosine kinase activity was displaced to the left compared with the curves mentioned above. These differences in dose–response curves cannot be attributed to the different muscle fibre composition, since purified insulin receptors from red and white skeletal muscle show identical

binding properties and similar sensitivities to insulin with regard to activation of insulin receptor kinase activity [33.34].

The differences in dose–response curves might be due to the existence of lower concentrations of insulin in the interstitial space of the intact muscle. However, our results with incubated intact muscle in the presence of 1 nM labelled insulin at 5°C indicate an almost complete equilibration of insulin between the incubation medium and the interstitial space. Although we are aware of the limitations of our experimental procedure for estimation of interstitial insulin (for instance, related to the low temperature during the incubation period), our data on interstitial insulin do not explain the differences found between the dose–response curves obtained in the intact muscle and in purified receptors.

Another explanation for these differences in the dose–response curves might relate to differences between insulin-binding affinities in the intact muscle (*in vivo* conditions, 37°C) and of partially purified insulin receptors (detergent-solubilized receptors, 25°C). In keeping with this view, it should be mentioned that: (*a*) partially purified insulin receptors from skeletal muscle show a greater insulin-binding affinity at 25°C than at 37°C (results not shown), and (*b*) when insulin receptors of rat skeletal muscle are partially purified by WGA affinity chromatography, this leads to a substantial decrease in the K_d for high-affinity insulin binding [41].

Based on our results and on previously published data describing a close correlation between insulin-stimulated glucose utilization and receptor kinase activity in skeletal muscle *in vivo* [42], we favour the existence of a general correlation between activation of receptor tyrosine kinase activity and insulin action *in vivo* in rat skeletal muscle. As a corollary of this, we have no evidence for the existence of spare tyrosine kinase activity in rat skeletal muscle. This is in contrast with data obtained in human muscle, in which the dose–response curve for insulin-stimulated glucose disposal was markedly displaced to the left compared with that for the activation of tyrosine kinase activity [43]. The reason for the differences between human and rat skeletal muscle is unknown.

In the present work we have also detected a clear parallel between the activation of insulin receptor tyrosine kinase activity and occupancy of high-affinity binding sites (high-affinity binding sites according to the two-site binding model, or high-affinity of empty sites according to the negative co-operativity model). In all, the pattern found in skeletal muscle indicates that: (*a*) full receptor kinase activation requires binding to all high-affinity binding sites, (*b*) maximal insulin action is obtained in skeletal muscle at insulin concentrations which allow full occupancy of high-affinity insulin receptors, and (*c*) there are no spare high-affinity insulin receptors. Furthermore, our results allow us to hypothesize that, in skeletal muscle, the low-affinity binding sites play no direct role in insulin action. This pattern found in skeletal muscle clearly differs from the one described in the adipocyte, i.e. the cell type in which the spare receptor concept has been clearly demonstrated. Thus 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 [26,27,44,45]. Therefore the insulin concentration required to stimulate the adipocyte half-maximally is lower than the dissociation constant for high-affinity binding. In this regard, the fact that the dose–response curve of insulin action in rat adipocytes is shifted to the left compared with the dose–response curve of insulin-stimulated receptor tyrosine kinase activity obtained both *in vivo* and *in vitro* [46–48] suggests that the spare receptor phenomenon of the rat adipocyte is due to post-receptor events. It should also be mentioned that intact adipose tissue shows a sensitivity to insulin which is relatively similar to the sensitivity found in intact muscle [49, 50]. Whether the spare receptor concept can be extrapolated to intact adipose tissue remains to be determined.

Based on the spare receptor concept, it was proposed that insulin-sensitivity can be modified in the isolated adipocyte either through modification of affinity binding characteristics or through changes in insulin receptor number, whereas insulin-responsiveness could be a consequence of changes at a post-binding level [51]. Nevertheless, on the basis of our data, which suggest the absence of spare high-affinity insulin receptors in skeletal muscle, we propose that modifications of insulin receptor affinity or number might have a different impact on insulin action in muscle. In consequence, the mechanisms by which insulin action is modified in skeletal muscle under physiological and pathological states deserve further re-evaluation. Thus, for example, the decreased number of insulin receptors reported in muscle from obese mice [52,53] might result in a diminished maximal response to insulin rather than in a lowered insulin sensitivity. Furthermore, insulin sensitivity might be altered in skeletal muscle by modification of insulin binding affinity, 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.

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