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The relationship between insulin binding, insulin activation of insulin-receptor tyrosine kinase, and insulin stimulation of glucose uptake in isolated rat adipocytes

Effects of isoprenaline

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We have studied the relationship between insulin activation of insulin-receptor kinase and insulin stimulation of glucose uptake in isolated rat adipocytes. Glucose uptake was half-maximally or maximally stimulated, respectively, when only 4% or 14% of the maximal kinase activity had been reached. To investigate this relationship also under conditions where the insulin effect on activation of receptor kinase was decreased, the adipocytes were exposed to 10 μM-isoprenaline alone or with 5 μg of adenosine deaminase/ml. An approx. 30% (isoprenaline) or approx. 50% (isoprenaline + adenosine deaminase) decrease in the insulin effect on receptor kinase activity was found at insulin concentrations between 0.4 and 20 ng/ml, and this could not be explained by decreased insulin binding. The decreased insulin-effect on kinase activity was closely correlated with a loss of insulin-sensitivity of glucose uptake. Moreover, our data indicate that the relation between receptor kinase activity and glucose uptake (expressed as percentage of maximal uptake) remained unchanged. The following conclusions were drawn. (1) If activation of receptor kinase stimulates glucose uptake, only 14% of the maximal kinase activity is sufficient for maximal stimulation. (2) Isoprenaline decreases the coupling efficiency between insulin binding and receptor-kinase activation, this being accompanied by a corresponding decrease in sensitivity of glucose uptake. (3) Our data indicate that the signalling for glucose uptake is closely related to receptor-kinase activity, even when the coupling efficiency between insulin binding and kinase activation is altered. They thus support the hypothesis that receptor-kinase activity reflects the signal which originates from the receptor and which is transduced to the glucose-transport system.

INTRODUCTION

The insulin receptor is a transmembrane glycoprotein composed of two α-subunits of 135 kDa and two β-subunits of 95 kDa [1,2]. Insulin binding to the α-subunits causes rapid phosphorylation of tyrosine residues on the β-subunits [1,3]. The phosphorylation of tyrosine-containing sites on the receptor in turn activates the insulin-receptor kinase toward tyrosine residues of other protein substrates [4–6]. Several lines of evidence indicate that autophosphorylation and activation of the insulin receptor as a tyrosine kinase is an important early step necessary for the transduction of many, if not all, of the biological effects of insulin [1,7,8]. A decreased ability of insulin receptors to autophosphorylate or to activate their kinase in response to insulin could therefore cause or contribute to insulin resistance. In fact, a decreased kinase activity of insulin receptors has been found in a variety of situations where the effects of insulin on biological actions are decreased [9–14], including type-II diabetes [15–17]. For technical reasons it has, however, so far been difficult to relate directly alterations in the coupling between insulin binding and insulin-induced activation of receptor kinase to alterations in biological actions such as glucose uptake. Firstly, in most studies, receptor kinase was not activated by stimulating intact cells with insulin, as was the case when biological actions were measured. Instead, receptors were first isolated, and only subsequently stimulated *in vitro* by insulin. The results may therefore not reflect the situation in the intact cell. Secondly, owing to the marked rightward shift of the concentration–response curve for insulin activation of receptor kinase compared with those for the major biological effects of insulin, it has been difficult to measure the kinase activity in an insulin concentration range where changes in insulin concentration lead, for example, to changes in glucose transport.

The aim of the present paper was to study the relationship between receptor kinase activity and glucose uptake at insulin concentrations which are sub-maximal or maximal for the stimulation of glucose uptake. Moreover, this relationship should be investigated under conditions where the coupling efficiency between insulin binding and activation of receptor kinase was altered. A highly sensitive assay to measure activation of insulin-receptor kinase in intact rat adipocytes was developed, such that insulin activation of receptor kinase, as well as 3-*O*-methylglucose uptake, could be measured under similar conditions and at similar insulin concentrations. Conditions with a decreased coupling efficiency between insulin binding and activation of receptor kinase were induced by preincubating the adipocytes with isoprenaline alone or together with adenosine deaminase (an enzyme which removes adenosine spontaneously released by isolated adipocytes from the incubation medium [18,19]). With this approach we tested the hypothesis that the signalling for glucose uptake is correlated with receptor-kinase activity rather than receptor occupancy under these conditions. In addition, we investigated how a decreased coupling efficiency between insulin binding and activation of receptor kinase may influence glucose uptake.

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MATERIALS AND METHODS

Materials

Pig monocomponent insulin was from Novo Biolabs (Bagsvaerd, Denmark) and [^{125}I-Tyr^{A14}]monoiodoinsulin from Amersham (Braunschweig, Germany). Serum from a patient with anti-insulin-receptor antibodies [20] was kindly supplied by Dr. Lawrence Mandarino (Pittsburgh, PA, U.S.A.). [γ-^{32}P]ATP (5000 Ci/mmol) and 3-*O*-methylglucose were purchased from New England Nuclear (Dreieich, Germany). Collagenase, BSA and histone 2B were from Boehringer Mannheim (Mannheim, Germany). Materials for SDS/PAGE were from Bio-Rad (München, Germany). D-Isopropylarterenol was from Serva (Heidelberg, Germany), and all other reagents were purchased from Sigma (München, Germany).

Isolation and incubation of cells

Adipocytes were prepared by collagenase digestion [21] of epididymal fat-pads from male Sprague–Dawley rats (150–180 g) fed *ad lib.* as described previously [19]. Incubations of the cells for measurement of insulin binding, 3-*O*-methylglucose uptake and activation of insulin-receptor kinase were performed under identical conditions. The cells were suspended in a buffer containing 128 mM-NaCl, 5.2 mM-KCl, 1.2 mM-$CaCl_2$, 1.3 mM-KH_2PO_4, 1 mM-Na_2HPO_4, 40 mM-Hepes, 3 mM-D-glucose and 2% BSA, pH 7.4. Cell concentration was (0.8–1.6) $\times 10^5$ cells/ml, temperature 37 °C, and the shaking speed 60/min in a rotatory water-bath. The cells were first incubated for 30 min at 37 °C in the absence or presence of 10 μM-isoprenaline. In some experiments, other substances (5 μg of adenosine deaminase/ml, 30 μM-propranolol or 1 μM-N^6-phenylisopropyladenosine) were added alone or in addition to isoprenaline. Subsequently, different amounts of insulin were added for an additional 15 min in order to measure insulin binding (mono[^{125}I]iodoinsulin), to activate the insulin-receptor kinase, or to stimulate glucose uptake, respectively.

Insulin binding to intact cells

This was determined with 0.2 ng of ^{125}I-insulin/ml and increasing concentrations of unlabelled insulin as previously described [19]. Incubations were ended after 15 min by separating the cells from the incubation buffer [19,22].

Activation of insulin-receptor kinase in intact cells

Measurement of insulin-receptor kinase activity after exposure of intact cells to insulin was performed essentially as previously described [19,23]; however, some modifications were introduced to increase the sensitivity of the assay. After incubation with insulin, cells were solubilized under conditions designed to preserve the phosphorylation state of the receptors as it existed in the intact cell [6,19,23]. This was achieved by addition of kinase, phosphatase and protease inhibitors during solubilization (1% Triton X-100, 2.5 mM-phenylmethanesulphonyl fluoride, 800 trypsin-inhibitor units of aprotinin/ml, 8 mM-EDTA, 160 mM-NaF, 10 mM-sodium pyrophosphate, 0.2 mM-sodium vanadate, 2 mM-dichloroacetic acid and 20 mM-Hepes, pH 7.4) and during partial purification of insulin receptors on wheat-germ agglutinin–agarose (0.05% Triton X-100, 100 mM-NaCl, 2.5 mM-KCl, 1 mM-$CaCl_2$, 0.1 mM-sodium vanadate, 100 mM-NaF, 10 mM-sodium pyrophosphate, 4 mM-EDTA, 10% glycerol and 20 mM-Hepes, pH 7.4). The insulin-binding activities of the wheat-germ agglutinin–agarose eluates were measured as previously described [11,19], and if necessary binding activities were adjusted by dilution. Samples of the eluates were then incubated for 16 h at 4 °C with Sepharose bearing anti-(insulin-receptor) antibody. At the end of this incubation period, 94–100% of the insulin receptors were bound to the antibody–Sepharose (as measured in control experiments using ^{32}P-labelled receptors). Isoprenaline and/or insulin had no effect on insulin-receptor binding to the antibody–Sepharose. The Sepharose was washed five times in buffer containing 0.05% Triton X-100, 100 mM-NaCl, 2.5 mM-KCl, 1 mM-$CaCl_2$, 0.1 mM-sodium vanadate and 20 mM-Hepes, pH 7.4, and resuspended in 30 μl of the same buffer. Phosphorylation assays were performed by addition of a mixture to give final concentrations of 5 mM-$MnCl_2$, 12 mM-$MgCl_2$, 500 μM-CTP, 1 mg of histone 2B/ml and 0.5 μM-[^{32}P]ATP (100–200 Ci/mmol). After 6 min at 4 °C (phosphate incorporation into histone was linear for more than 20 min), reactions were terminated by adding Laemmli buffer [24] and heating to 95 °C for 5 min [19]. Phosphorylated proteins were analysed by SDS/PAGE on 15%-acrylamide gels.

3-*O*-Methylglucose uptake

Glucose uptake was assayed by measuring initial rates of 3-*O*-methylglucose influx after rapid concentration of the cells (to $\sim 10^6$ cells/ml) by centrifugation at the end of the incubations with insulin. All data presented are measurements of zero-*trans* influx, which was measured in quadruplicate determinations by a modification of the method of Whitesell & Gliemann [25]. The portion of cellular uptake owing to diffusion and trapping of the label in the extracellular space was measured by performing parallel reactions with L-[1-^{14}C]glucose as substrate. All results for glucose uptake are corrected for the L-glucose value.

Cellular ATP

This was measured by a modification of an established method [26]. Briefly, 50 μl of the incubation medium containing $\sim$ 5000 cells was added to 100 μl of ice-cold 10% trichloroacetic acid and 0.15% of the detergent SB14. Samples were immediately vortex-mixed, and after 5 min then centrifuged. Then 20 μl of the supernatant was added to 500 μl of 0.1 M-Tris/KOH buffer (pH 8), and the ATP content was rapidly measured with luciferase reagent.

RESULTS

Effect of isoprenaline alone or plus adenosine deaminase on insulin binding

In the absence of adenosine deaminase, isoprenaline (10 μM) had no effect on insulin binding to the adipocytes (Fig. 1). Only when adenosine deaminase (5 μg/ml) was added in addition to isoprenaline, a small decrease of insulin binding ($\sim$ 20%) was observed. As alterations in the pH of the incubation medium can influence insulin binding, we have measured the pH of the medium at the end of the incubations. Under the three different conditions, the pH fell from 7.4 at the beginning of the incubations to 7.38$\pm$0.01 (no additions), 7.37$\pm$0.01 (isoprenaline) and 7.35$\pm$0.01 (isoprenaline and adenosine deaminase).

Insulin binding to wheat-germ-agglutinin-purified insulin receptors extracted from cells incubated with or without isoprenaline (or isoprenaline + adenosine deaminase) was also measured. No effect of isoprenaline or adenosine deaminase on the number or insulin affinity of the receptors was found (results not shown).

Effect of isoprenaline alone or plus adenosine deaminase on insulin-dependent activation of insulin-receptor kinase in intact cells

The effect of incubation of the cells with insulin on activation of insulin-receptor kinase is shown in Figs. 2(*a*) and 2(*b*). This

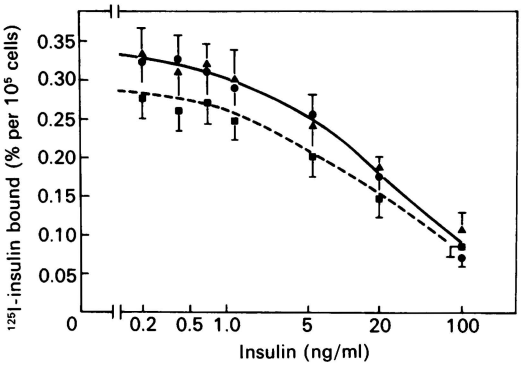

Fig. 1. Effect of isoprenaline (alone or plus adenosine deaminase) on insulin binding to isolated adipocytes

Adipocytes were incubated for 30 min in the absence (●) or presence of isoprenaline alone (▲) or plus adenosine deaminase (■) as described in the Materials and methods section. Subsequently ^{125}I-insulin (0.2 ng/ml) and unlabelled insulin at the indicated concentrations were added and incubations continued for 15 min. Incubations were ended by separating the cells from the incubation buffer as previously described [22]. The values are corrected for non-specific binding and represent the means ± S.E.M. of 12 independent experiments with triplicate determinations.

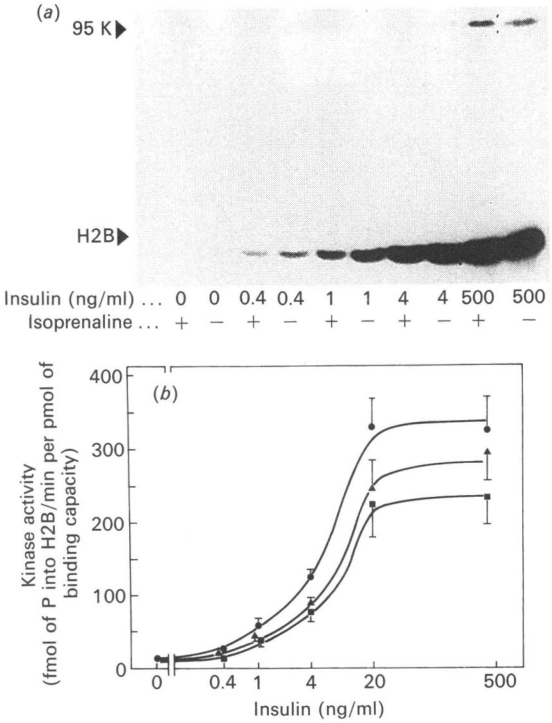

Fig. 2. Insulin activation of insulin-receptor kinase in intact adipocytes after incubation in the absence (●) or presence of isoprenaline alone (▲) or plus adenosine deaminase (■)

Adipocytes were incubated for 30 min with or without the indicated substances under the same conditions as for the measurement of insulin binding. Subsequently insulin (0–500 ng/ml) was added and incubations continued for 15 min. At the end of incubations, cells were rapidly solubilized in the presence of protease, kinase and phosphatase inhibitors. Insulin-receptor kinase activity was measured as described in the Materials and methods section. (*a*) An autoradiograph showing phosphorylation of histone 2B (H2B) in a representative experiment (95 K: phosphorylated 95 kDa β-subunit of insulin receptor). (*b*) Dose–response curves for insulin activation of insulin-receptor kinase in intact cells. The values represent means ± S.E.M. of 5–10 independent experiments with duplicate determinations.

incubation of the cells stimulated insulin-receptor kinase ~ 25-fold, and the low background allowed measurements at insulin concentrations as low as 0.4 ng/ml. Insulin activation of insulin-receptor kinase was significantly decreased by isoprenaline at all sub-maximal insulin concentrations measured ($P < 0.05$; Student paired *t* test). In the presence of isoprenaline, approximately twice the insulin concentration was necessary to evoke similar kinase activities as in the absence of isoprenaline. This effect of isoprenaline was more pronounced when adenosine was depleted from the medium with adenosine deaminase (Fig. 2*b*). To test the specificity of the effect of isoprenaline and adenosine deaminase, propranolol (30 μM) or N^6-phenylisopropyladenosine (1 μM) respectively were added in addition to isoprenaline or adenosine deaminase in some experiments. Propranolol abolished the effects of isoprenaline, and N^6-phenylisopropyladenosine (an adenosine analogue that is not deaminated by adenosine deaminase) the effects of adenosine deaminase (results not shown).

Effect of isoprenaline alone or plus adenosine deaminase on 3-*O*-methylglucose uptake

The data in Fig. 3 show that isoprenaline led to a decrease in the maximal insulin effect on 3-*O*-methylglucose uptake as well as to a shift to the right of the concentration–response curve [EC_{50} (half-maximally effective concn.) 0.4 or 0.6 ng of insulin/ml in the absence or presence of isoprenaline respectively]. Under conditions where adenosine was depleted from the incubation buffer by adenosine deaminase, these effects of isoprenaline were more pronounced (EC_{50} 1 ng of insulin/ml).

Relationship between insulin-receptor kinase activity and glucose uptake

The concentration–response curves for activation of receptor kinase and for glucose uptake were quite different. Although 3-*O*-methylglucose uptake was already maximal at an insulin concentration of 1 ng/ml (Fig. 3, ●), activation of insulin-receptor kinase at this concentration accounted for only 14% of the maximum (Fig. 2*b*, ●). To investigate the relationship between receptor-kinase activity and glucose uptake further, insulin stimulation of glucose uptake (expressed as percentage of

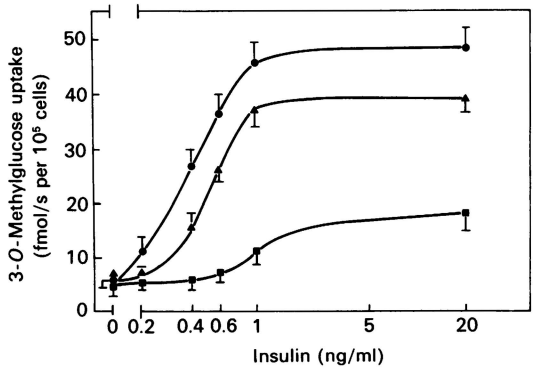

Fig. 3. Insulin stimulation of 3-*O*-methylglucose uptake in adipocytes after incubation in the absence (●) or presence of isoprenaline alone (▲) or plus adenosine deaminase (■)

Adipocytes were incubated for 30 min with or without isoprenaline alone or plus adenosine deaminase, as described above. Subsequently insulin was added at the indicated concentrations, and incubations continued for 15 min. Initial rates of 3-*O*-methylglucose uptake were determined as specified in the Materials and methods section. Data represent means of quadruplicate determinations ± S.E.M. of 5 individual experiments.

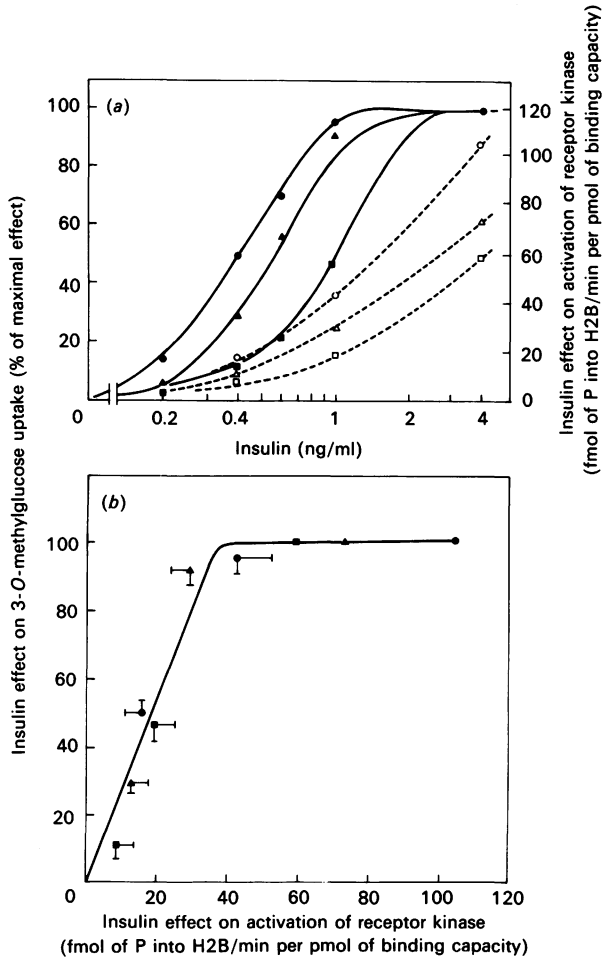

Fig. 4. Relation between activity of receptor kinase and glucose uptake

(*a*) Comparison of the effects of insulin to activate insulin-receptor kinase and to stimulate glucose uptake. In this Figure, the data for insulin-stimulated glucose uptake are shown as percentages of the maximal glucose uptake (●, no additions; ▲, in the presence of isoprenaline; ■, in the presence of isoprenaline + adenosine deaminase). Additionally, the lower part of the dose–response curve for insulin activation of insulin-receptor kinase is depicted (○, no additions: △, in the presence of isoprenaline; □, in the presence of isoprenaline and adenosine deaminase). Data represent the mean values of results obtained in the same experiments as shown in Figs. 2 and 3. (*b*) Data for insulin-stimulated glucose uptake (expressed as percentage of the maximal uptake) are plotted against the activity of receptor kinase measured under similar conditions (●, no additions; ▲, with isoprenaline; ■, with isoprenaline + adenosine deaminase) and similar insulin concentrations (0.4, 1 and 4 ng/ml). Values represent means ± S.E.M.

the maximal effect) and insulin activation of receptor kinase are depicted together in Fig. 4(*a*). The data show that similar kinase activities correspond to similar glucose uptake (expressed as percentage of maximal transport) under all conditions tested. For example, at a kinase activity of 18 fmol/min per pmol binding capacity a 50% stimulation of glucose uptake is observed, regardless of whether 0.4, 0.6 or 1.0 ng of insulin/ml is necessary to elicit this kinase activity in control cells, isoprenaline-treated cells or cells treated with isoprenaline and adenosine deaminase respectively. At a kinase activity of ~ 40 fmol/min per pmol maximal glucose uptake is reached under all conditions tested. The data indicate that the relation between insulin-receptor kinase activity and glucose uptake (the latter expressed as percentage of maximal glucose uptake) remains constant, even under conditions where the coupling efficiency between insulin binding and activation of insulin-receptor kinase is altered. This is also demonstrated in Fig. 4(*b*), where glucose-uptake data are plotted against receptor-kinase activity.

Effect of isoprenaline alone or plus adenosine deaminase on cellular ATP levels

Because alterations in cellular ATP concentration may influence insulin activation of insulin-receptor kinase, cellular ATP levels were measured at the end of the incubations in four independent experiments. The data showed that ATP levels were slightly decreased by the incubations with isoprenaline alone or together with adenosine deaminase. In the absence of insulin, ATP concentrations were 771 ± 133, 738 ± 43 and 712 ± 92 pmol/10^5 adipocytes respectively, with no additions, or in the presence of isoprenaline alone or together with adenosine deaminase. In the presence of insulin (500 ng/ml), ATP concentrations were 802 ± 95, 680 ± 59 and 659 ± 58 pmol/10^5 adipocytes respectively under the three conditions.

DISCUSSION

In the present study, concentration–response curves for insulin activation of insulin-receptor tyrosine kinase and for insulin activation of glucose uptake in isolated rat adipocytes have been compared. Our data show that if activation of insulin-receptor kinase represents the intracellular signal which leads to a stimulation of glucose uptake, only 14% of the maximal kinase activity is sufficient for a maximal stimulation (Figs. 2*b* and 3).

To investigate possible consequences of alterations in the coupling between insulin binding and activation of receptor kinase for glucose uptake, the cells were exposed to isoprenaline (alone or plus adenosine deaminase). These substances clearly decreased the coupling efficiency between insulin binding and the activation of receptor kinase. Thus isoprenaline had no effect on insulin binding (Fig. 1), but under the same conditions significantly decreased the insulin effect on the activation of receptor kinase (Fig. 2). When adenosine deaminase was present in addition to isoprenaline, the decrease in the coupling efficiency between insulin binding and activation of receptor kinase was more pronounced than with isoprenaline alone.

These alterations in the coupling efficiency between insulin binding and activation of receptor kinase induced by isoprenaline (alone or plus adenosine deaminase) were accompanied by a decrease in the insulin-sensitivity of glucose uptake. Moreover, a quantitative analysis of the data revealed that, although isoprenaline (alone or plus adenosine deaminase) altered the coupling efficiency between insulin binding and activation of receptor kinase, the relationship between receptor-kinase activity and glucose uptake (expressed as percentage of maximal glucose uptake) remained unchanged (Figs. 4*a* and 4*b*). Thus a given kinase activity corresponded to similar glucose uptake (expressed as percentage of maximal uptake), regardless of whether this kinase activity was reached in the absence of isoprenaline (alone or plus adenosine deaminase), or in their presence together with a correspondingly higher insulin concentration, i.e. a higher fractional receptor occupancy. These findings indicate that the signalling for glucose uptake is more closely related to receptor-kinase activity than to receptor occupancy under these conditions. They thus support the hypothesis that insulin-receptor kinase activity reflects the intracellular signal which originates from the insulin receptor and which is transduced to the glucose-transport system. Moreover, these data suggest that a decreased coupling efficiency between insulin binding and activation of insulin-receptor kinase results in a decreased insulin-

sensitivity of glucose uptake. With regard to the mechanism of isoprenaline-induced insulin resistance, our results indicate that the decreased insulin-sensitivity of glucose uptake in the presence of isoprenaline (alone or plus adenosine deaminase) can be fully accounted for by the decreased activation of receptor kinase.

Although our data suggest that the decrease in insulin-receptor kinase activity accounts for the decreased insulin-sensitivity of glucose uptake in the presence of isoprenaline (alone or plus adenosine deaminase), it is not clear what causes the decrease in the maximal glucose uptake (Fig. 3). The most likely explanation is that, in addition to its effects on the coupling between insulin binding and receptor kinase, isoprenaline has effects on the transduction of the insulin signal distal to insulin-receptor kinase, or has effects on the glucose-transport system itself. The latter notion is supported by studies by James *et al.* [27], who found that isoprenaline induces phosphorylation of the insulin-regulatable glucose transporter in rat adipocytes. This may decrease its intrinsic ability to transport glucose [27,28].

Our data on the effects of isoprenaline on insulin binding confirm findings by Arsenis & Livingston [12], who found that isoprenaline has no effect on insulin binding when a fall in pH owing to free fatty acid release is prevented by appropriate assay conditions. Only when adenosine deaminase was added in addition to isoprenaline did we observe a small decrease in insulin binding to the cells. Possibly this was caused by the slightly lower pH in the incubation buffer under this condition. In any event, this small decrease in insulin binding could not explain the decreased insulin effect on receptor-kinase activity. The effect of isoprenaline on kinase activity of insulin receptors from rat adipocytes has previously been investigated by Häring *et al.* [13] and Arsenis & Livingston [12]. In contrast with our present paper, insulin receptors were first isolated and then stimulated with insulin in a cell-free system in both studies. Changes in the receptor-kinase activity detected by that method may not reflect the situation in the intact cell. Our data on the effect of isoprenaline on glucose transport are consistent with previous reports [12,29–31].

The mechanism by which isoprenaline decreases the coupling between insulin binding and activation of insulin-receptor kinase in intact rat adipocytes is not clear. Previous studies suggest a possible role of phosphorylation of serine residues of the insulin receptor [14,32,33]. Another possible mechanism in intact cells could have been decreased cellular ATP levels, possibly in combination with an increased K_m for ATP of the receptors [13]. Chung *et al.* [34] reported that, in the absence of glucose in the incubation buffer, β-adrenergic agonists extensively deplete ATP in rat adipocytes via conversion into cyclic AMP. Under our conditions (3 mM-glucose), however, cellular ATP levels were decreased by less than 20% under all conditions tested.

The observation that in rat adipocytes an increase in receptor-kinase activity is coupled to an increase in glucose uptake only up to a certain point (Figs. 4*a* and 4*b*) suggests that there is a 'rate-limiting step' in the transduction of the insulin signal distal to the kinase activation. The biochemical basis of this may be a limited amount of a signalling compound that is phosphorylated by the insulin receptor or interacts with it in some other way in order to transmit the insulin signal. Alternatively it is possible that the effector systems themselves (like the glucose-transport system) can only react to the insulin signal up to a certain intensity. In any event, as a consequence of the 'spare' kinase, one would expect that a decreased ability of insulin receptors to activate their kinase in response to insulin will result in decreased insulin-sensitivity, rather than a decreased responsiveness, of glucose uptake. This notion is supported by our results, which show a close correlation between the effects of isoprenaline (alone or plus adenosine deaminase) to decrease the insulin-sensitivity of glucose uptake and their effects to decrease the coupling efficiency between insulin binding and insulin activation of receptor kinase. It remains, however, to be established whether a similar relationship between the insulin-sensitivity of glucose uptake and an altered ability of insulin receptors to activate their kinase in response to insulin is also present in pathophysiological states of insulin resistance like type-2 diabetes mellitus.

In summary, we have demonstrated in isolated rat adipocytes that, if activation of receptor kinase leads to a stimulation of glucose uptake, only 14% of the maximal kinase activity is sufficient for maximal stimulation. Under conditions with decreased coupling efficiency between insulin binding and activation of receptor kinase, a corresponding decrease in insulin-sensitivity of glucose uptake was observed. Our data thus lend support to the hypothesis that insulin-receptor kinase activity reflects the intracellular signal which originates from the insulin receptor and which is transduced to the glucose-transport system.

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