Biochemical Pharmacology, Vol. 38, No. 14, pp. 2269–2277, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

QUANTITATIVE DISSOCIATION OF GLUCOSE TRANSPORT STIMULATION AND INSULIN RECEPTOR TYROSINE KINASE ACTIVATION IN ISOLATED ADIPOCYTES WITH A COVALENT INSULIN DIMER (B29,B29′-SUNBEROYL-INSULIN)

HANS G. JOOST,*† RÜDIGER GÖKE,† CHRISTOPH SCHMITZ-SALUE,† HANS J. STEINFELDER† and DIETRICH BRANDENBURG‡

†Institute of Pharmacology und Toxicology, University of Göttingen, Robert-Koch-Straße 40, D-3400 Göttingen, Federal Republic of Germany, and ‡Deutsches Wollforschungsinstitut, Veltmanplatz 8, D-5100 Aachen, Federal Republic of Germany

(*Received* 15 *September* 1988; *accepted* 18 *January* 1989)

Abstract—The covalent insulin dimer B29,B29′-suberoyl-insulin was investigated for its effects on insulin receptor binding, insulin receptor tyrosine kinase activity and glucose transport in isolated adipose cells. The dimer stimulated glucose transport (initial 3-*O*-methylglucose uptake rate) to the same extent as insulin did (basal rate, 35 ± 3 pmol/sec/μl lipid; insulin, 380 ± 27; B29,B29′-suberoyl-insulin, 369 ± 24, means ± S.E.), although at higher concentrations (EC_{50} 1.94 ± 0.64 nM versus 0.1 ± 0.02 with insulin). In contrast, the dimer only partially (23%) mimicked insulin's effect on phosphate incorporation into insulin receptors immunoprecipitated after equilibration of cells with [^{32}P]phosphate. Similarly, insulin receptor tyrosine kinase as assessed by receptor autophosphorylation and phosphorylation of the substrate poly-(Glu/Tyr) was not fully activated by treatment of cells with the insulin dimer (31 and 42% of the effect of insulin, respectively) in concentrations which maximally activate glucose transport and give rise to full insulin receptor occupancy (5×10^{-7} M). Further, the dimer activated the receptor tyrosine kinase in solubilized purified insulin receptor preparations from adipose cells to only 25% of the effect of insulin (EC_{50} 32.0 ± 16 versus 1.9 ± 1.0 nM with insulin) in spite of full receptor occupancy. Binding of the dimer to insulin receptors followed single site binding kinetics, indicating that the derivative is unable to induce negative cooperativity of the insulin receptor. It is concluded that a partial phosphorylation of insulin receptors and a submaximal tyrosine kinase activation are sufficient for full stimulation of glucose transport in the adipocyte. Further, it is suggested that negative cooperativity of the insulin receptor and activation of its tyrosine kinase require a similar conformational change of the receptor protein.

It is generally accepted that the diverse actions of insulin are initiated by binding of the hormone to a single insulin receptor [1]. This receptor possesses an intrinsic protein kinase activity which catalyses autophosphorylation of its β-subunit, as well as phosphorylation of exogenous substrates [2–4]. Activation of the receptor kinase is the most rapid event following binding of insulin to the receptor [5] and might be the unique signalling mechanism for all actions of insulin.

Recently, site directed insulin receptor mutants lacking the tyrosine phosphorylation sites [6] or the ATP-binding site [7] have been inserted into insulin-sensitive cells. In these cell lines, the effect of insulin on 2-deoxyglucose transport was impaired in parallel to the tyrosine kinase inactivation. Further, injection of a monoclonal antibody, which specifically inhibited the tyrosine kinase activity of the receptor, into Chinese hamster ovary cells inhibited insulin's effect on 2-deoxyglucose transport, phosphorylation of ribosomal protein S6 and glycogen synthesis [8]. It appears reasonable to conclude on the basis of these data that the tyrosine kinase activation of the insulin receptor is required for most, if not all, metabolic effects of insulin.

However, the latter conclusion may be questioned on the basis of two reports dissociating the receptor kinase from the acute metabolic effects of insulin [9, 10]. A monoclonal antibody to the insulin receptor which antagonized receptor kinase activity was recently reported [10] to exert full insulinomimetic effects on glucose transport in human adipocytes. Further, a polyclonal antiserum to the insulin receptor with insulin-like efficacy stimulated glucose transport and insulin receptor internalization but not receptor autophosphorylation in adipocytes [9]. However, antiserum from the same patient (B10) has very recently been described to stimulate receptor autophosphorylation and tyrosine kinase activity as well as 2-deoxyglucose transport in Chinese hamster ovary cell [11].

A third approach to correlate or differentiate effects of insulin consists of the use of insulin derivatives. Insulin analogues and derivatives, naturally occurring as well as chemically modified ones, usually exhibit a close relation between binding affinity to the insulin receptor and potency of their metabolic effects [12]. A few derivatives have been reported to

* Address correspondence and reprint requests to: Hans G. Joost, Institute of Pharmacology and Toxicology, Robert-Koch-Straße 40, D-3400 Göttingen, F.R.G.

deviate from this rule in that their binding affinity relative to their metabolic activity is greater than that of the 'normal' insulin derivatives [12]. Agents with such characteristics are potential antagonists, thereby allowing the classic pharmacological approach to differentiate separate pathways of action. Among the derivatives which showed a disproportionate relation between receptor binding and biological effect, the covalently dimerized insulins exhibited the largest difference between binding and action [13, 14]. The insulin derivative covalently dimerized with a suberoyl chain at the B29 lysine (B29,B29'-suberoyl-insulin) produced a very low stimulation of the receptor kinase (1% of the effect of insulin) in IM9 lymphocytes but triggered the same internalization of insulin receptors as did insulin [15].

Thus, in the present study the effects of the dimerized insulin derivative on the receptor kinase activity were compared to those on glucose transport in isolated adipocytes which exhibit a large response to the hormone. The data show that the covalent insulin dimer B29,B29'-suberoyl-insulin is a full agonist in stimulating glucose transport. In contrast, the derivative only partially stimulates insulin receptor autophosphorylation and receptor tyrosine kinase in intact adipocytes or in a purified receptor preparation. It is concluded, therefore, that the acute effect of insulin on glucose transport in adipocytes requires only a partial activation of the insulin receptor tyrosine kinase.

EXPERIMENTAL PROCEDURES

Preparation of B29,B29'-suberoyl-insulin. Bovine insulin was converted to its A1,B1-methylsulfonylethoxycarbonyl derivative, cross-linked between the B29 lysine groups with suberic *bis*(*p*-nitrophenyl) ester, and was unblocked as described [16]. The purity of the covalent dimer was checked with high-performance liquid chromatography and no monomeric insulin was detected.

Animals and cell preparation. Male Wistar rats, weighing 160–220 g, bred in our institute were used throughout. Adipose cells were isolated from epididymal adipose tissue by collagenase digestion as described [17] with minor variations [18].

Incubation of cells and determination of glucose transport activity. All incubations were carried out at 37° in a KRBH* buffer [18], pH 7.4, containing 4% bovine albumin (Fraction V, Serva Chemicals, Heidelberg, F.R.G.) which was purified with charcoal prior to use, 1 mM glucose and 200 nM adenosine [19]. In the experiments designed to study phosphate incorporation, the total phosphate concentration was lowered to 0.1 mM, and sodium [^{32}P]phosphate was added (0.2–0.4 mCi/ml). Cells were allowed to equilibrate with the tracer for 90 min, and were thereafter exposed to insulin or the insulin dimer for 30 min. Glucose transport activity in isolated adipose cells was assayed with the non-metabolizable 3-*O*-methylglucose [20] with modifications previously described in detail [18]. Transport was measured at 37° with a pulse time of 3 sec and initial uptake rates were calculated from the uptake values U_t and U_{max} [21]. In order to determine the maximum uptake, cells were incubated with the label for 60 min.

Preparation of plasma membranes. Isolated adipocytes were homogenized as previously described in detail [21, 22] in a buffer containing Tris (20 mM), sucrose (255 mM), PMSF (1 mM) and phosphatase inhibitors, namely, sodium fluoride (10 mM), sodium pyrophosphate (20 mM) and sodium vanadate (0.2 mM). Plasma membranes were isolated by differential centrifugation as described previously [21, 22].

Immunoprecipitation of insulin receptor. Plasma membranes (approximately 1 mg of protein) were solubilized for 30 min on ice in a buffer containing (mM): Hepes, 50, NaCl, 150, NaF, 10, sodium pyrophosphate, 20, sodium vanadate, 0.2, and 1% Triton X-100. Samples were centrifuged (15,000 *g*, 30 min) and the antiserum B10 (1:100) was added to the supernatants. After 16–24 hr, immunocomplexes were separated with protein A sepharose, washed 3 times with buffer containing Hepes (50 mM), NaCl (150 mM) and Triton X-100 (0.1%) and eluted with electrophoresis sample buffer. Samples were separated by SDS-PAGE, and gels were stained, dried and autoradiographed for 1–3 weeks.

Preparation of partially-purified insulin receptor preparation from adipocyte plasma membranes. Solubilized plasma membranes (200–400 μl) were added to a suspension of 100 μl wheat germ agglutinin agarose (Miles Laboratory, München, F.R.G.) and incubated for 60 min on ice. The beads were separated by centrifugation (2000 rpm, 2 min), and the supernatant was aspirated and discarded. After 3 washes with buffer containing Hepes (50 mM), NaCl (150 mM) and Triton X-100 (0.1%), the partially-purified receptor was eluted with 200–400 μl of washing buffer supplemented with 0.3 M *N*-acetylglucosamine. Binding was assayed and the eluates were, if necessary, concentrated by centrifugation in Amicon centricon tubes. Fractions containing insulin binding activity were frozen in liquid nitrogen and were stored at −70° until used.

Assay of tyrosine kinase activity. Tyrosine kinase activity of insulin receptor was assayed with the synthetic substrate poly-(Glu/Tyr) (Sigma Chemicals, St. Louis, MO) essentially as described previously [23]. Aliquots (40 μl containing 50–100 fmol of insulin binding activity) of the partially-purified receptor preparation were incubated with or without insulin or the insulin dimer at 20° for 20 min. Reaction buffer (100 μl) containing 75 mM Hepes, 1.5 mM $MnCl_2$, 15 mM $MgSO_4$, 1.5 mM CTP, 0.5% Triton X-100 and 15 μg/ml albumin was added. The reaction was started by addition of 5 μl of a solution containing the exogenous substrate Poly-(Glu/Tyr) to a final concentration of 0.3 mg/ml and [^{32}P]ATP to a final concentration of 50 μM (approximately 0.5 μCi/sample) and was terminated after incubation at 20° for 10 min by applying duplicate aliquots (70 μl) onto cellulose filter papers (Whatman 3MM).

* Abbreviations used: KRBH, Krebs–Ringer bicarbonate HEPES buffer; PMSF, phenylmethylsulfonylfluorid; poly-(Glu/Tyr), co-polymerized glutamic acid/tyrosine 4:1.

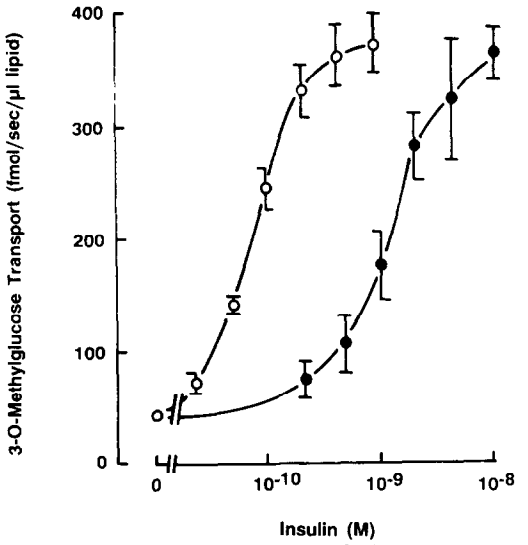

Fig. 1. Concentration dependency of glucose transport activation by insulin and B29,B29′-suberoyl-insulin in isolated adipocytes. Isolated adipocytes were incubated with the indicated concentrations of insulin (open circles) or the insulin dimer (filled circles) at 37° for 30 min, and 3-*O*-methylglucose transport was assayed as described under Materials and Methods. The data represent means ± SE of four separate experiments.

The filters were immediately immersed in 10% tricholoroacetic acid containing 10 mM sodium pyrophosphate, extensively washed, dried and the radioactivity was determined with the aid of a toluene-based scintillation cocktail. Blanks run in parallel contained less than 0.03% of the total radioactivity. Phosphate incorporation into the substrate was linear over a period of 20 min.

Autophosphorylation of the partially-purified insulin receptor. The partially-purified insulin receptor (20 μl with approximately 200 fmol of binding activity) was incubated as indicated with or without insulin or the insulin dimer for 20 min at 20°. Reaction buffer (50 μl, see above) was added, and the phosphorylation was started by addition of 5 μl of [^{32}P]ATP (1–2 μCi/sample) to a final concentration of 50 μM. The reaction was stopped after 15 min by addition of 25 μl of a solution containing 10 mM EDTA, 20 mM sodium pyrophosphate, 20 mM ATP, 250 mM Tris, 200 mM dithiothreitol, 5% SDS, 25% glycerol and 0.02% Bromophenol Blue. The samples were heated to 95° for 2 min and applied to a vertical slab gel electrophoresis (stacking gel 3.5%, resolving gel 7%). The gels were stained with Coomassie Brilliant Blue, destained, dried and autoradiograms were prepared. Films were exposed (Agfa Curix RP 1 film) for 1–4 days at −70° with the aid of an intensifying screen.

Determination of insulin binding. Isolated adipocytes were treated with 2 mM KCN for 5 min in order to block the internalization of insulin receptors, and ^{125}I-(A14)-insulin (40 pM, 10–20 nCi/sample) together with the desired concentrations of unlabeled insulin or the insulin dimer was added. Samples were incubated at room temperature for another 30 min, and cells were separated by centrifugation through silicone oil. Samples of plasma membranes (40 μg protein/sample) were incubated at 4° overnight with tracer insulin and unlabeled insulin, and the membranes were separated by centrifugation (15,000 *g*) in a refrigerated microfuge. Tracer binding in the presence of 10^{-6} M insulin (non-specific binding) was less than 5% of tracer bound in the absence of hormone. Solubilized insulin receptor was incubated with tracer insulin and unlabeled insulin or the insulin dimer at 4° overnight and the bound fraction was separated by precipitation with polyethylene glycol 6000 (13% w/v final concentration) in the presence of 0.5 mg/ml gamma-globulin as carrier protein [23].

Calculations. Statistical significance was tested with a paired *t*-test, and differences were accepted as significant at the $P < 0.05$ level. Insulin binding curves were evaluated with the IBM-PC version of the LIGAND (SCAFIT) program [24] (courtesy of Dr P. J. Munson, National Institutes of Health, Bethesda, MD).

RESULTS

Stimulation of glucose transport by insulin and B29,B29′-suberoyl-insulin

As has been shown previously for their effect on

Table 1. Relative potencies of insulin and B29,B29′-suberoyl-insulin in stimulating glucose transport and inhibiting insulin receptor binding in isolated adipocytes, and stimulating tyrosine kinase activity in partially purified insulin receptor from adipocyte plasma membranes

		Insulin	B29,B29′-suberoyl-insulin
EC_{50} of methylglucose transport stimulation in adipocytes (nM)		0.1 ± 0.02	1.94 ± 0.64
	(1) K_D:	0.29 ± 0.1	
K_D of ^{125}I-insulin binding in adipocytes (nM)	(2) K_D:	8.20 ± 1.6	5.07 ± 1.2
R_0 (total binding sites) of insulin binding in adipocytes (fmol/10^6 cells)		130.4 ± 10	141.2 ± 26
EC_{50} of tyrosine kinase activation in partially purified receptor from adipocyte plasma membranes (nM)		1.93 ± 1.03	32.0 ± 16

EC_{50}s of methylglucose transport stimulation were determined by graphical evaluation of a series of experiments run in parallel with the binding experiments and performed like those shown in Fig. 1. Binding data were obtained from the curves shown in Fig. 2 with the LIGAND/SCAFIT program [24]. Displacement of ^{125}I-(A14)-insulin with insulin was evaluated on the basis of a two-site model. Displacement with the insulin dimer essentially followed a single site kinetic and was evaluated accordingly. EC_{50}s of tyrosine kinase activation were obtained by graphical evaluation of the experiments shown in Fig. 3. The data represent means ± SE of four separate experiments.

lipogenesis in isolated adipocytes [14], both insulin and the insulin dimer produced the same stimulatory effect on methylglucose transport (Fig. 1). Initial uptake rates were 35 ± 3 fmol/sec/μl lipid in basal adipocytes and maximally stimulated rates were 380 ± 27 in insulin-treated cells and 369 ± 24 in adipocytes stimulated with the insulin dimer. However, as is illustrated in Fig. 1, the potency of the insulin dimer as judged from the half maximally effective concentrations was much lower than that of insulin. Concentrations of insulin and the insulin dimer producing half maximal transport stimulation differed by a factor of 19 (Table 1).

Inhibition of insulin receptor binding by insulin and B29,B29'-suberoyl-insulin

As is illustrated in Fig. 2, the dimerized insulin derivative was about one order of magnitude less potent in inhibiting ^{125}I-insulin binding in adipose cells as well as in isolated plasma membranes. A computerized evaluation of the binding curves (Table 1) revealed that the insulin dimer, in contrast to insulin, inhibited tracer binding according to a single site model. As judged from the K_D values, the affinity of binding of the insulin dimer was 18-fold lower than the high affinity binding of insulin (Table 1). Further, the evaluation showed an essentially identical number of sites on the basis of molar concentrations of the agents, indicating that only one insulin moiety of the dimer combines with the receptor.

In vitro *stimulation of the insulin receptor kinase by insulin and B29,B29'-suberoyl-insulin*

In partially-purified insulin receptor preparations from adipocyte plasma membranes, insulin produced a large increase in phosphate incorporation into the synthetic substrate poly-(Glu/Tyr) (Fig. 3). In contrast, concentrations of the cross-linked insulin derivative which produce full receptor occupancy and glucose transport stimulation activated the tyrosine kinase of the receptor to a considerably lesser extent (approximately 25% of the effect of insulin). The concentrations of insulin and the insulin dimer giving rise to a half maximal stimulation of the tyrosine kinase (Table 1) differed by a factor of 17 which is essentially identical with that observed for the differences in glucose transport stimulation and insulin receptor binding (Table 1). Like the phosphorylation of the exogenous substrate poly-(Glu/Tyr), autophosphorylation of the β-subunit of the solubilized receptor preparation in response to the insulin dimer was much lower than in response to insulin (Fig. 4).

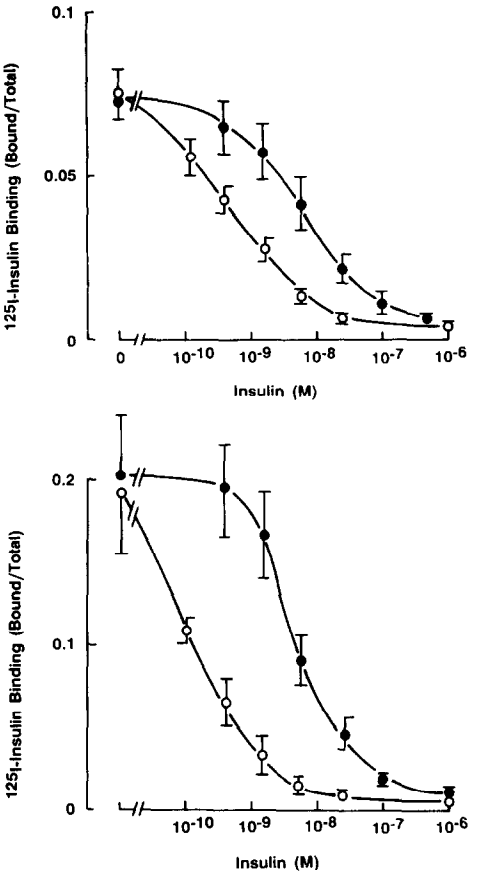

Fig. 2. Displacement curves of tracer insulin binding inhibited by insulin or B29,B29'-suberoyl-insulin in isolated adipocytes and adipocyte plasma membranes. Upper panel: Isolated adipocytes (samples of approximately 2 × 10^5 cells/200 μl) were incubated with 2 mM KCN for 5 min, and with ^{125}I-insulin (final concentration 40 pM) and the indicated concentrations of unlabeled insulin (open circles) and the insulin dimer (filled circles) for additional 40 min. Cells were separated as described under Materials and Methods by the oil flotation method and cell associated radioactivity was determined. The data represent means ± SE of three different experiments and were not corrected for non-specific binding. Binding characteristics as determined with the LIGAND/SCAFIT program are given in Table 1. Lower panel: Plasma membranes from basal adipocytes (40 μg protein/200 μl) were incubated in the presence of ^{125}I-insulin (final concentration 40 pM) and the indicated concentrations of unlabeled insulin (open circles) and B29,B29'-suberoyl-insulin (filled circles) for 16 hr at 4°. Membranes were separated from incubation buffer by centrifugation, and membrane-associated radioactivity was determined. The data represent means ± SE and were not corrected for non-specific binding.

In vivo *stimulation of insulin receptor phosphorylation by insulin and B29,B29'-suberoyl-insulin*

Since the effects of insulin on a solubilized receptor (*in vitro*) may differ from those produced by treatment of intact cells (*in vivo*), the effects of the insulin dimer on the receptor kinase were studied in intact cells (*in vivo*) and compared to those of insulin. Adipose cells were equilibrated with [^{32}P]phosphate, subsequently treated with insulin or the insulin dimer and a membrane fraction was prepared from which insulin receptors were isolated by immunoprecipitation. Figure 5 shows the [^{32}P]phosphate incorporation into the β-subunit of the insulin receptor in response to insulin and the insulin dimer. The figure depicts the autoradiogram of the immunoprecipitates separated on SDS-PAGE, and the subsequent quantitation of phosphate incorporation by cutting and counting of the 95 kDa band. The figure illustrates that insulin produced a substantial increase of

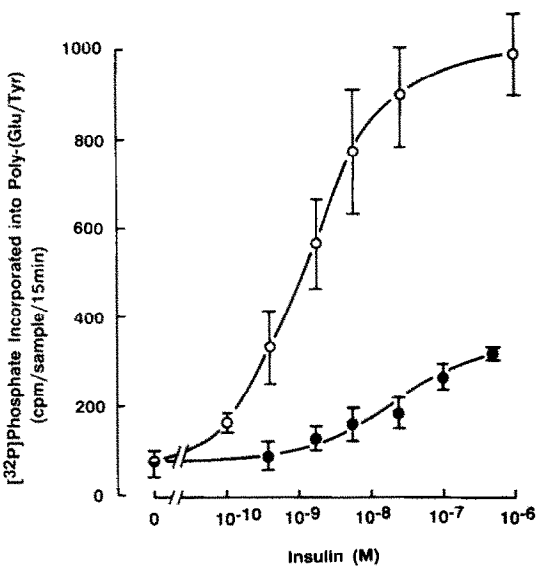

Fig. 3. Concentration dependency of tyrosine kinase activation in partially purified insulin receptor from adipocyte plasma membranes (*in vitro*) by insulin or B29,B29'-suberoyl-insulin. Insulin receptor was partially purified from adipocyte plasma membranes by wheat-germ agglutinin affinity chromatography as described in Materials and Methods. Approximately 40 fmol of insulin binding activity per sample were incubated with the indicated concentrations of insulin (open circles) or the insulin dimer (filled circles) at 22° for 30 min. The reaction was started by addition of the substrate and [^{32}P]ATP, and was terminated after 15 min. The data represent means ± SE of 4 experiments.

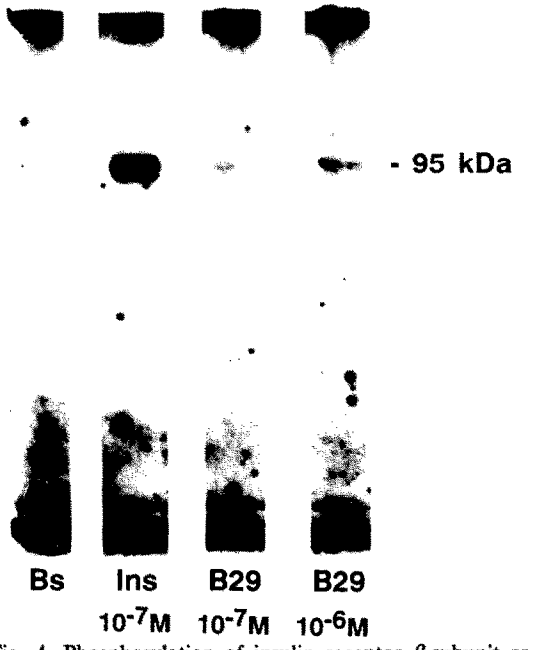

Fig. 4. Phosphorylation of insulin receptor β-subunit as stimulated by insulin or B29,B29'-suberoyl-insulin in partially-purified insulin receptor (*in vitro*) from adipocyte plasma membranes. Insulin receptor was solubilized and partially purified from adipocyte plasma membranes as described under Materials and Methods. Samples were incubated with the indicated concentrations of insulin and B29,B29'-suberoyl-insulin for 20 min, followed by phosphorylation with [^{32}P]ATP for 15 min, and were separated on SDS-PAGE. The figure shows the autoradiograph of a representative experiment.

[^{32}P]phosphate incorporation into the 95 kDa band (about 3-fold). Although the insulin dimer gave rise to a significant [^{32}P]phosphate incorporation into the receptor (23% of the effect of insulin), the dimerized insulin clearly failed to maximally activate receptor autophosphorylation in concentrations (5×10^{-7} M) which give rise to full receptor occupancy and glucose transport stimulation.

In vivo *stimulation of insulin receptor kinase activity by insulin and B29,B29'-suberoyl-insulin*

In a parallel series of experiments carried out with unlabeled adipocytes, isolated cells were treated with the agents, and a solubilized receptor preparation was prepared and assayed for its activity to phosphorylate the synthetic substrate poly-(Glu/Tyr) (Fig. 6, upper panel) or the receptor β-subunit (Fig. 6, lower panel). In order to normalize the data for receptor concentration, insulin binding in the receptor preparations from plasma membranes was assayed. As was expected [25], insulin binding was lower in membranes from insulin-treated cells (59.2 ± 5.1% of the basal value). Treatment of cells with the insulin dimer produced a significant, but smaller decrease in insulin binding in plasma membranes (76.7 ± 8.3% of the basal value).

As Fig. 6 illustrates, insulin treatment produced a 3.8-fold increase of phosphorylation of the synthetic substrate (upper panel). A similar, although smaller increase was observed, when phosphorylation of the β-subunit of the receptor was assayed (lower panel). Again, the insulin dimer failed to elicit the full insulin response on the receptor tyrosine kinase activity in a concentration which gives rise to full receptor occupancy and glucose transport stimulation; the stimulatory effect of the insulin dimer on receptor autophosphorylation and poly-(Glu/Tyr) phosphorylation was 31 and 42% of the effect of insulin, respectively.

DISCUSSION

The covalently cross-linked insulin derivative B29,B29'-suberoyl-insulin produced the same stimulatory effect as insulin on glucose transport, but gave rise to a considerably lower effect than insulin on receptor phosphorylation and tyrosine kinase activity of the insulin receptor *in vivo* as well as *in vitro*. Thus, the data indicate a quantitative dissociation of glucose transport stimulation and receptor kinase activation in adipocytes. Based on the assumption that insulin receptor phosphorylation and tyrosine kinase activation initiate the acute effects of insulin on glucose transport, the present data indicate that a submaximal phosphorylation of the receptor is sufficient for maximal transport activation.

Convincing evidence for a role of the tyrosine

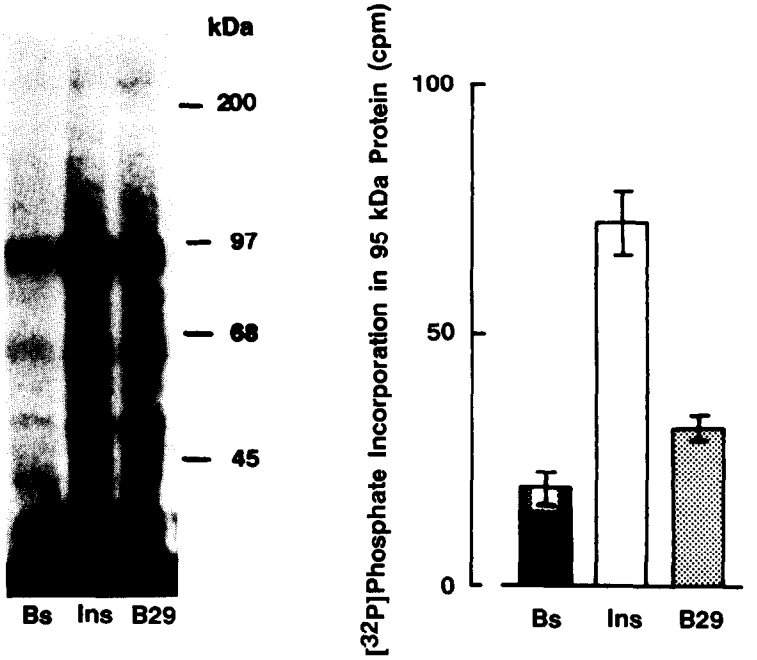

Fig. 5. Phosphorylation of insulin receptor β-subunit as stimulated by insulin or B29,B29'-suberoyl-insulin in isolated adipocytes (*in vivo*). Isolated adipocytes were prelabeled with [^{32}P]phosphate (0.25 mCi/ml) for 90 min. Cells were exposed to insulin (0.5 μM) or the insulin dimer (0.5 μM) for 30 min. Plasma membranes were isolated and solubilized as described under Materials and Methods, and insulin receptors were immunoprecipitated with the B10 serum. Control samples immunoprecipitated with normal human serum were run in parallel and did not show any radioactivity in the 95 kDa region. The figure shows the autoradiograph of a representative experiment, and the quantitation of radioactivity incorporated into the 95 kDa band as means ± SE of a series of 3 separate experiments.

kinase in the acute effects of insulin has been presented recently [6–8]. In contrast, a complete dissociation of glucose transport stimulation and insulation receptor kinase activation has previously been reported on the basis of experiments with a polyclonal [9] or a monoclonal antibody [10] to the insulin receptor. These antibodies produced a full stimulation of glucose transport but no stimulation of insulin receptor phosphorylation in human [10] or rat adipocytes [9]. In an attempt to reconcile the divergent findings, it might be postulated that glucose transport in the adipocyte differs from that in the mutant cell lines by a component which is not mediated by the tyrosine kinase activation. Alternatively, it appears possible that the receptor kinase activation in response to the antibodies was too small to be detectable but sufficient to produce transport stimulation. On the basis of the present findings, no more than a portion of 30% of insulin's effect on receptor phosphorylation is required for full glucose transport stimulation. Judged from the low effect of insulin in the studies with the receptor antiserum on receptor autophosphorylation and receptor kinase activity (1.7-fold in [9] and 1.3-fold in [10]), a 10–20% stimulation of the receptor kinase might have been sufficient to mediate full transport stimulation.

The present results suggest that a substantial part of the total insulin receptor autophosphorylation and tyrosine kinase activity does not participate in mediating insulin's effect on glucose transport. Thus, the heterogeneity of insulin receptor phosphorylation has to be considered as an explanation of the present findings. In the solubilized receptor preparation, insulin stimulates autophosphorylation exclusively on tyrosine residues. In contrast, if intact cells are prelabeled with [^{32}P]phosphate and subsequently treated with insulin, an additional increase in serine phosphorylation of the receptor is observed [26]. Moreover, peptide mapping of the phosphorylated receptor revealed striking differences in the phosphorylation sites depending on whether the receptor was labeled in a cell-free system or in the intact cell [27]. Therefore, in intact cells insulin appears to stimulate a serine kinase in addition to the activation of the receptor tyrosine kinase [28]. It is interesting to note that very recently two forms of insulin receptors were identified in hepatoma cells by sequential immunoprecipitation with anti-phosphotyrosine and anti-insulin receptor antibodies [29]. About 80% of the total receptors were precipitated with anti-phosphotyrosine antibodies and contained mostly phosphotyrosine, whereas the remaining 20% contained mainly phosphoserine and phosphothreonine [29]. Finally, activating and non-activating components of receptor autophosphorylation have been distinguished recently on the basis of kinetic data [30]. In view of the present results it might be speculated on a functional heterogeneity of the phosphorylated insulin receptor mediating different effects of insulin in target cells.

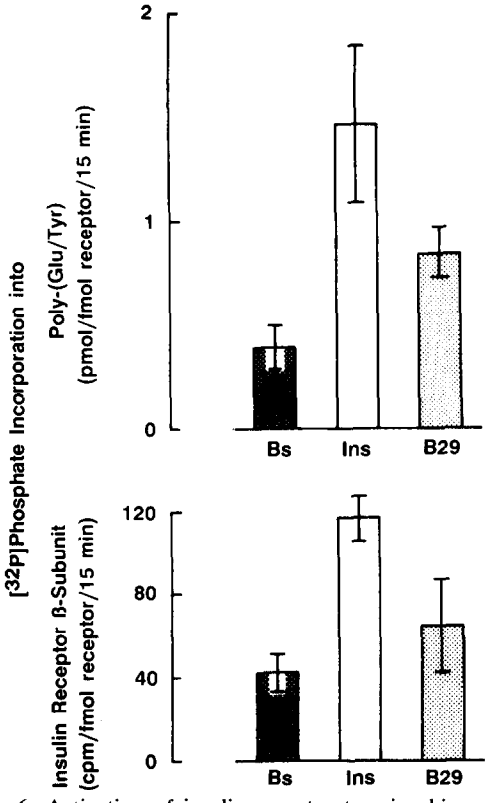

Fig. 6. Activation of insulin receptor tyrosine kinase in isolated adipocytes (*in vivo*) by insulin or B29,B29′-suberoyl-insulin. Isolated adipocytes were incubated in the presence of insulin or the insulin dimer (0.1 μM) for 30 min. Plasma membranes were isolated, solubilized, and insulin receptor was partially purified as described under Materials and Methods. Insulin binding was determined, and tyrosine kinase activity was assayed with exogenous substrate (upper panel). In a separate series, samples were incubated for 15 min with [^{32}P]ATP in the absence of exogenous substrate, separated by electrophoresis, and the radioactivity incorporated into the 95 kDa band was determined and normalized for insulin binding. Binding data are given in the text. Data represent means ± SE of 3 separate experiments.

According to the present data the cross-linked insulin derivative B29,B29′-suberoyl-insulin is a partial agonist of the insulin receptor tyrosine kinase. In concentrations which gave rise to full receptor occupancy, the compound exhibited a considerably lower intrinsic activity than insulin as stimulator of the receptor kinase. Partial agonists usually show antagonistic properties in that they inhibit the effect of a full agonist. Indeed, the insulin dimer inhibited the effect of small insulin concentrations on the receptor tyrosine kinase in solubilized insulin receptor preparations from human placenta and adipocyte membranes (manuscript in preparation).

It has been suggested that B29,B29′-suberoyl-insulin, in contrast to other covalently cross-linked insulin derivatives, might bivalently bind to the receptor [31]. However, on the basis of a model calculation for bivalent ligands a much higher binding affinity would be expected, if one molecule of the insulin dimer saturated both α-subunits of the receptor [13]. Further, evaluation of our binding data (R_0) suggest a stoichiometric inhibition of insulin binding by the insulin dimer and supports a monovalent binding of the dimer, leaving one insulin moiety unable to engage in receptor binding.

The present data indicate that the insulin dimer binds with lower affinity than insulin to insulin receptors. These data are in good agreement with a study in mouse adipocytes (K_D = 8.3 nM for the insulin dimer; 0.13 and 14 nM for insulin [32]). Other reports [13, 15] indicated that the insulin dimer inhibits ^{125}I-insulin binding with essentially the same affinity as insulin. It is generally accepted, however, that the insulin dimer binds to insulin receptors with a single K_D. Scatchard analysis of our data (Fig. 2) confirmed that binding of the insulin dimer to adipocytes and adipocyte membranes can be described with a single site model, whereas insulin binding produced curvilinear Scatchard plots. If a two site model is assumed to account for the curvilinearity of insulin binding, the dimer would bind to both sites with the same, low affinity. Alternatively, if negative cooperativity of the receptor is assumed [33], the dimer may lack the potency to induce conformational changes of the receptor which in turn decrease its affinity. Accordingly, covalently dimerized insulin derivatives have previously been described to inhibit the accelerating effect of insulin on the dissociation of receptor bound ^{125}I-insulin [31]. Interestingly, recent evidence emphasized the crucial role of the α-subunit interaction by showing that negative cooperativity requires a tetrameric receptor ($\alpha_2\beta_2$) [34].

In contrast to its effect on glucose transport, the insulin dimer only partially stimulated tyrosine kinase activity of the insulin receptor in concentrations which gave rise to full receptor occupancy. The intramolecular signal transduction from the binding site (extracellular α-subunit) to the tyrosine kinase (β-subunit, intracellular side of the membrane) is a transmembrane signalling process and does probably require a profound conformational alteration of the receptor. Like the negative cooperativity of the receptor, the tyrosine kinase activation is abolished when the tetrameric receptor is converted to an αβ-dimer, and therefore appears to depend on the interaction of the α-subunits [35]. It is conceivable that the insulin dimer hinders the conformational alterations of the receptor, possibly α-subunit interaction, which activate the tyrosine kinase. Since there is a striking parallel between the reduced tyrosine kinase activation and the lack of negative cooperativity in response to the insulin dimer, it is tempting to speculate that these effects require a common conformational change of the insulin receptor.

Acknowledgements—The authors wish to thank Dr A. Schüttler and C. Brandenburg for the preparation of the insulin dimer, Dr S. Taylor for a gift of the insulin receptor antiserum (B10), Drs T. M. Weber and S. DiPaolo for helpful suggestions for the tyrosine kinase assay of insulin receptor from adipose cells and G. Degenhardt for graphics and photography. This study has been supported by grants from the Deutsche Forschungsgemeinschaft and the Juvenile Diabetes Foundation.

REFERENCES

1. Czech MP, The nature and regulation of the insulin receptor: Structure and function. *Annu Rev Physiol* **47**: 357–381, 1985.
2. Kasuga M, Karlsson FA and Kahn CR, Insulin stimulates the phosphorylation of the 95,000-dalton subunit of its own receptor. *Science* **215**: 185–187, 1982.
3. Petruzzelli L, Herrera R and Rosen OM, The insulin receptor is an insulin-dependent tyrosine protein kinase: Copurification of insulin binding activity and protein kinase activity to homogeneity from human placenta. *Proc Natl Acad Sci USA* **81**: 3327–3331, 1984.
4. Kohansky RA, Frost SC and Lane MD, Insulin-dependent phosphorylation of the insulin receptor-protein kinase and activation of glucose transport in 3T3-L1 adipocytes. *J Biol Chem* **261**: 12272–12281, 1986.
5. Pang DT, Sharma BR, Shafer JA, White MF and Kahn CR, Predominance of tyrosine phosphorylation of insulin receptors during initial response of intact cells to insulin. *J Biol Chem* **260**: 7131–7136, 1985.
6. Ellis L, Clauser E, Morgan DO, Edery M, Roth RA and Rutter WJ, Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose. *Cell* **45**: 721–732, 1986.
7. Chou CK, Dull TJ, Russell DS, Gherzi R, Lebwohl D, Ullrich A and Rosen OM, Human insulin receptors mutated at the ATP-binding site lack protein tyrosine kinase activity and fail to mediate postreceptor effects of insulin. *J Biol Chem* **262**: 1842–1847, 1987.
8. Morgan DO and Roth RA, Acute insulin action requires insulin receptor kinase activity: Introduction of an inhibitory monoclonal antibody into mammalian cells blocks the rapid effects of insulin. *Proc Natl Acad Sci USA* **84**: 41–45, 1987.
9. Simpson IA and Hedo JA, Insulin receptor phosphorylation may not be a prerequisite for acute insulin action. *Science* **223**: 1301–1304, 1984.
10. Forsayeth JR, Caro JF, Sinha MK, Maddux BA and Goldfine ID, Monoclonal antibodies to the human insulin receptor that activate glucose transport but not insulin receptor kinase activity. *Proc Natl Acad Sci USA* **84**: 3448–3451, 1987.
11. Gherzi R, Russel DS, Taylor SI and Rosen OM, Reevaluation of the evidence that an antibody to the insulin receptor is insulinomimetic without activating the protein tyrosine kinase activity of the receptor. *J Biol Chem* **262**: 16900–16905, 1987.
12. Gammeltoft S, Insulin receptors: Binding kinetics and structure-function relationship of insulin. *Physiol Rev* **64**: 1321–1378, 1984.
13. Tatnell MA, Jones RH, Willey KP, Schuettler A and Brandenburg D, Evidence concerning the mechanism of insulin–receptor interaction and the structure of the insulin receptor from biological properties of covalently linked insulin dimers. *Biochem J* **216**: 687–694, 1983.
14. Willey KP, Tatnell MA, Jones RH, Schuettler A and Brandenburg D, Biological properties of covalent insulin dimers. In: *Insulin. Chemistry, Structure and Function of Insulin and Related Hormones* (Eds. Brandenburg D and Wollmer A), pp. 425–431. Walter de Gruyter, Berlin, New York, 1980.
15. Roth RA, Cassell DJ, Morgan DO, Tatnell MA, Jones RH, Schuettler A and Brandenburg D, Effects of covalently linked insulin dimers on receptor kinase activity and receptor down regulation. *FEBS Lett* **170**: 360–364, 1984.
16. Schüttler A and Brandenburg D, Preparation and properties of covalently linked insulin dimers. *Hoppe-Seyler's Z Physiol Chem* **363**: 317–330, 1964.
17. Rodbell M, Metabolism of isolated fat cells: Effects of hormones on glucose metabolism and lipolysis. *J Biol Chem* **239**: 375–380, 1964.
18. Joost HG, Steinfelder HJ, Strodt J and Wehmeyer J, Modulation of glucose transport in hamster adipocytes by insulin and by beta- and alpha2-adrenoceptor agonists. *Diabetologia* **29**: 371–377, 1986.
19. Honnor RC, Dhillon GS and Londos C, cAMP-dependent protein kinase and lipolysis in rat adipocytes. I. Cell preparation, manipulation and predictability in behavior. *J Biol Chem* **260**: 15122–15129, 1985.
20. Whitesell RR and Gliemann J, Kinetic parameters of transport of 3-*O*-methylglucose and glucose in adipocytes. *J Biol Chem* **254**: 5276–5283, 1979.
21. Weber TM, Joost HG, Simpson IA and Cushman SW, Methods for assessment of glucose transport activity and the number of glucose transporters in isolated rat adipose cells and membrane fractions. In: *Receptor Biochemistry and Methodology. Insulin Receptors Part B: Biological Responses, and Comparison to the IGF-I Receptor* (Eds. Kahn GR and Harrison L), pp. 171–187. A.R.Liss, New York, 1988.
22. Joost HG, Weber TM and Cushman SW, Qualitative and quantitative comparison of glucose transport activity and glucose transporter concentration in plasma membranes from basal and insulin-stimulated rat adipose cells. *Biochem J* **249**: 155–161, 1988.
23. Joost HG, Steinfelder HJ and Schmitz-Salue C, Tyrosine kinase activity of insulin receptors from human placenta: Effects of autophosphorylation and cyclic AMP-dependent protein kinase. *Biochem J* **233**: 677–681, 1985.
24. Munson PJ and Rodbard D, Ligand: A versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem* **107**: 220–239, 1980.
25. Wang CC, Sonne O, Hedo JA, Cushman SW and Simpson IA, Insulin-induced internalization of the insulin receptor in the isolated rat adipose cell. Detection of the internalized 138 kDa receptor subunit using a photoaffinity ^{125}I-insulin. *J Biol Chem* **258**: 5129–5134, 1983.
26. Kasuga M, Zick Y, Blith DL, Karlsson FA, Haering HU and Kahn CR, Insulin stimulation of phosphorylation of the beta-subunit of the insulin receptor. Formation of both phosphoserine and phosphotyrosine. *J Biol Chem* **257**: 9891–9894, 1982.
27. White MF, Takayama S and Kahn CR, Differences in the sites of phosphorylation of the insulin receptor *in vivo* and *in vitro*. *J Biol Chem* **260**: 9470–9478, 1985.
28. Yu KT, Khalaf N and Czech MP, Insulin stimulates a membrane-bound serine kinase that may be phosphorylated on tyrosine. *Proc Natl Acad Sci USA* **84**: 3972–3976, 1987.
29. White MF, Maron R and Takayama S, The function of insulin receptors in hepatoma cells. *Diabetes* **35**(Suppl. 1): 2A, 1986.
30. Kohansky RA and Lane MD, Kinetic evidence for activating and non-activating components of autophosphorylation of the insulin receptor protein kinase. *Biochem Biophys Res Commun* **134**: 1312–1318, 1986.
31. Piron MA, Michiels-Place M, Waelbroeck M, De Meyts P, Schuettler A and Brandenburg D, Structure-activity relationships of insulin-induced negative cooperativity among receptor sites. In: *Insulin. Chemistry, Structure and Function of Insulin and Related Hormones* (Eds. Brandenburg D and Wollmer A), pp. 371–392. Walter de Gruyter, Berlin, New York, 1980.
32. Schlüter K, Petersen K-G, Schuettler A, Brandenburg D and Kerp L, Biological activity and receptor binding of six different covalent dimers of insulin. In: *Insulin. Chemistry, Structure and Function of Insulin and Related Hormones* (Eds. Brandenburg D and Wollmer A), pp. 433–438. Walter de Gruyter, Berlin, New York, 1980.
33. DeMeyts P, Bianco AR and Roth J, Site-site interactions among insulin receptors. Characterization of

the negative cooperativity. *J Biol Chem* **251**: 1877–1888, 1976.
34. Deger A, Kraemer H, Rapp R, Koch R and Weber U, The nonclassical insulin binding of insulin receptors from rat liver is due to the presence of two interacting alpha-subunits in the receptor complex. *Biochem Biophys Res Commun* **135**: 458–464, 1986.
35. Boeni-Schnetzler M, Rubin JB and Pilch PF, Structural requirements for the transmembrane activation of the insulin receptor kinase. *J Biol Chem* **261**: 15281–15287, 1986.