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Antagonistic effects of a covalently dimerized insulin derivative on insulin receptors in 3T3-L1 adipocytes

(glucose transport/DNA synthesis)

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ABSTRACT **In the present study we describe the antagonistic effects of the covalently dimerized insulin derivative B29,B29′-suberoyl-insulin on insulin receptors in 3T3-L1 mouse cells. In differentiated 3T3-L1 adipocytes, the derivative fully inhibits binding of ^{125}I-labeled insulin to its receptor with about the same affinity as unlabeled insulin. In contrast, the dimerized derivative only partially (approximately 20%) mimics insulin's effects on glucose transport and DNA synthesis in the absence of insulin. In the presence of insulin, the agent competitively inhibits insulin-stimulated DNA synthesis ([^{3}H]thymidine incorporation into total DNA), glucose transport activity (2-deoxyglucose uptake rate), and insulin receptor tyrosine kinase activity. In rat adipocytes, in contrast, the dimerized derivative stimulates glucose transport (initial 3-*O*-methylglucose as well as 2-deoxyglucose uptake rates) to the same extent as insulin does, and it fails to inhibit the effect of insulin. The data indicate that the dimerized insulin derivative B29,B29′-suberoyl-insulin is an insulin receptor antagonist (partial agonist) which retains a moderate intrinsic activity. The effects of this agent reveal a striking difference in insulin receptor-mediated stimulation of glucose transport between 3T3-L1 fatty fibroblasts and the mature rat adipocyte.**

A large number of insulin analogues from different species and synthetically modified derivatives have previously been tested for their biological activity (1). All derivatives investigated so far were pure agonists of the insulin receptor, since they differed only in potency (concentration eliciting half-maximal effects, EC_{50}) but produced identical maximal responses in various bioassays. Accordingly, an excellent correlation between the binding affinity of the derivatives and their biological potency in the fat cell bioassay was observed. As a notable exception from this correlation, insulins from hagfish, porcupine, and coypu had lower biological activities than expected on the basis of their binding affinities (1). Further, dimerized insulin derivatives exhibited an even more striking discrepancy between binding affinity and biological potency (2). In IM9 lymphocytes, the covalently dimerized insulin derivative B29,B29′-suberoyl-insulin induced insulin receptor internalization to the same extent as insulin but failed to stimulate the receptor tyrosine kinase (3). In isolated adipocytes, the compound fully stimulated glucose transport but produced only 25% of the maximal effect of insulin on the receptor tyrosine kinase activity under conditions of full receptor occupancy (4, 5). These data prompted us to assume that dimerized insulin derivatives might antagonize some actions of insulin and might therefore be used to analyze diverse effects of the hormone. Thus, in the present study we investigated the effects of the covalent insulin dimer B29,B29′-suberoyl-insulin on DNA synthesis and glucose transport in 3T3-L1 adipocytes, initially aiming at a differentiation of the acute effects (metabolism) and the long-term effects (mitogenesis) of the hormone. The data presented in this study indicate that B29,B29′-suberoyl-insulin is indeed an insulin receptor antagonist. The derivative competitively inhibited all investigated effects of insulin in the 3T3-L1 cell but was a pure agonist of glucose transport stimulation in the rat fat cell.

MATERIALS AND METHODS

Preparation of B29,B29′-Suberoyl-Insulin. B29,B29′-suberoyl-insulin was synthesized as described previously (6). Briefly, bovine insulin was converted to its A1,B1-methylsulfonylethoxycarbonyl derivative and crosslinked between the B29 lysine groups with suberic bis(*p*-nitrophenyl) ester, and the product was unblocked. The purity of the covalent dimer was checked with high-performance liquid chromatography, and no monomeric insulin was detected.

Cell Culture. 3T3-L1 fatty fibroblasts (7) were obtained from the American Type Culture Collection (Rockville, MD) and were grown in Dulbecco's modified Eagle's medium containing 10% newborn calf serum, 20 mM Hepes, and 2 mM glutamine. Cells were induced to differentiate 2 days after confluence (7 days after plating) with a modification of the described protocol (8) by treatment with isobutylmethylxanthine (0.5 mM), dexamethasone (0.25 μM), and insulin (1 μg/ml) for 3 days followed by treatment with insulin alone for 4 days. During differentiation, media were supplemented with fetal calf serum (10%) instead of newborn calf serum.

Isolation of Adipocytes. 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 (9) with minor variations (10). All incubations of fat cells were carried out at 37°C in Krebs–Ringer bicarbonate–Hepes buffer, pH 7.4, containing 4% bovine albumin (fraction V, Serva), 1 mM glucose, and 200 nM adenosine (11).

Assay of Binding of Insulin and Insulin-Like Growth Factor I (IGF-I). Insulin radioiodinated at the A14 tyrosine (^{125}I-insulin) and radioiodinated IGF-I (^{125}I-IGF-I) were purchased from Amersham-Buchler. Unlabeled IGF-I was a generous gift from W. Märki (CIBA–Geigy, Basel, Switzerland). Differentiated 3T3-L1 cells were incubated at 4°C in a Krebs–Ringer Hepes buffer containing 2% albumin, ^{125}I-insulin or ^{125}I-IGF-I (20 nCi; 1 nCi = 37 Bq), and the indicated concentrations of unlabeled ligand. After an overnight incubation, the buffer was removed and cells were washed three times with ice-cold buffer and lysed with 0.1% sodium dodecyl sulfate, and cell-associated radioactivity was determined.

Insulin Receptor Autophosphorylation. Differentiated 3T3-L1 cells were homogenized with a Potter–Elvehjem homog-

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Abbreviation: IGF-I, insulin-like growth factor I.

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enizer and were centrifuged at 100,000 × *g* for 60 min. The resulting pellet was solubilized in 50 mM Hepes buffer, pH 7.4, containing 1% Triton X-100, and the solubilized insulin receptors were partially purified by absorption to wheat germ agglutinin agarose (12). Samples of the receptor were incubated with insulin and B29,B29′-suberoyl-insulin for 20 min, and autophosphorylation was initiated by addition of [γ-^{32}P]ATP (2 μCi per sample, total ATP concentration 50 μM), in the presence of Mg^{2+} and Mn^{2+} as described (12). The reaction was terminated by addition of electrophoresis sample buffer containing dithiothreitol (200 mM) and sodium dodecyl sulfate (2 mM), and the mixture was subsequently boiled for 2 min. After electrophoresis, phosphate incorporation into the 95-kDa subunit of the insulin receptor was visualized by autoradiography of the dried gels.

Glucose Transport Assays. Glucose transport activity in isolated adipose cells was assayed with the nonmetabolizable glucose analogue 3-*O*-methylglucose (13) with modifications previously described in detail (14). In addition, glucose transport activity was determined in 3T3-L1 adipocytes with 2-[^{3}H]deoxyglucose as described (8). Differentiated 3T3-L1 cells were incubated for 2 hr in serum-free culture medium supplemented with 1% bovine serum albumin. Thereafter, the cells were washed three times with 1.5 ml of Krebs–Ringer Hepes buffer containing 0.2% albumin and were incubated for another 15 min in the same buffer supplemented with 0.2% bovine serum albumin and the indicated concentrations of insulin and B29,B29′-suberoyl-insulin. The transport assay was started by addition of 2-[^{3}H]deoxyglucose (1 μCi per well) to a final concentration of 0.1 mM. Uptake was allowed to proceed for 10 min at 37°C and was terminated by cooling the cells on ice. The buffer was removed and cells were washed three times with ice-cold incubation buffer. The cells were lysed with 0.1% sodium dodecyl sulfate, and radioactivity incorporated was determined by scintillation counting in a water-compatible scintillation cocktail. The cell count was determined with parallel samples, and data were normalized per number of cells.

Determination of DNA Synthesis. Differentiated 3T3-L1 adipocytes were washed once with Krebs-Ringer Hepes buffer (0.2% albumin), incubated for 48 hr in serum- and insulin-free medium supplemented with 1% albumin, and exposed thereafter to the indicated concentrations of insulin and/or B29,B29′-suberoyl-insulin for 17.5 hr. Thereafter, cells were incubated with [^{3}H]thymidine (1 μCi per well) for 2 hr. Thymidine incorporation was terminated by three washes with ice-cold buffer, and cells were lysed in 0.1 M sodium hydroxide. Lysates were precipitated with trichloroacetic acid (20%) and filtered with suction through glass fiber filters (GF 103; Schleicher & Schüll). The filters were rinsed five times with ice-cold trichloroacetic acid (10%) and once with ethanol. After drying, tritium incorporation into the filtered material was assayed by liquid scintillation counting.

RESULTS

In 3T3-L1 adipocytes, B29,B29′-suberoyl-insulin stimulated a moderate increase in both glucose transport activity and DNA synthesis but failed to elicit the maximal insulin response of either effect (Fig. 1). The stimulation of DNA synthesis by both insulin and the dimerized derivative consisted of two components, one of which (low-EC_{50}) was probably mediated through the insulin receptor, the other one (high-EC_{50}) through the IGF-I receptor. The relative potency of the dimer in stimulating the second, presumably IGF-I receptor-mediated, component was much lower than that of insulin.

Insulin binding to 3T3-L1 adipocytes was fully inhibited by B29,B29′-suberoyl-insulin (Fig. 2). The affinity of the dimer to the insulin receptor appeared similar to, if not higher than,

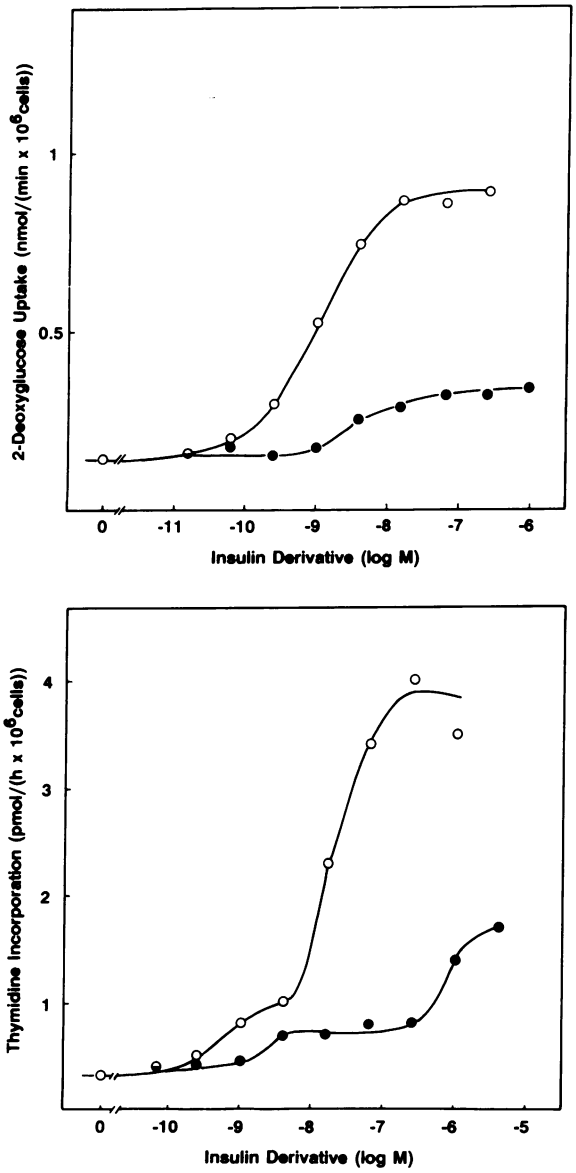

FIG. 1. Effects of B29,B29′-suberoyl-insulin on glucose transport and DNA synthesis in 3T3-L1 adipocytes. (*Upper*) Differentiated 3T3-L1 adipocytes were incubated for 2 hr in serum-free medium, washed with Krebs–Ringer Hepes buffer, and exposed to the indicated concentrations of insulin (○) or B29,B29′-suberoyl-insulin (●) in Krebs–Ringer Hepes buffer for 15 min. Thereafter, 2-deoxyglucose uptake was assayed. All incubations were performed at 37°C. (*Lower*) Differentiated 3T3-L1 adipocytes were cultured in serum-free medium for 48 hr and were exposed to the indicated concentrations of insulin (○) or B29,B29′-suberoyl-insulin (●) for 17.5 hr. Thereafter the cells were incubated with [^{3}H]thymidine for 120 min, and incorporation of tritium into total DNA was assayed. The data represent means of triplicate samples from a representative experiment.

that of insulin itself. As is illustrated in Fig. 2, B29,B29′-suberoyl-insulin inhibited the binding of ^{125}I-insulin to 3T3-L1 adipocytes with a slightly higher potency than unlabeled insulin. Higher concentrations of the dimer than of insulin were required to significantly inhibit IGF-I binding (Fig. 2 *Lower*), as can be expected from the lower potency of the dimer in stimulating the high-EC_{50} component of DNA synthesis (Fig. 1 *Lower*). Thus, the affinity of B29,B29′-suberoyl-insulin to the IGF-I receptor appears to be lower than that of insulin. Taken together, the data shown in Figs. 1 and 2 indicate that the dimer acted as a partial agonist of the insulin receptor with a high binding affinity and a low intrinsic activity (agonist efficacy).

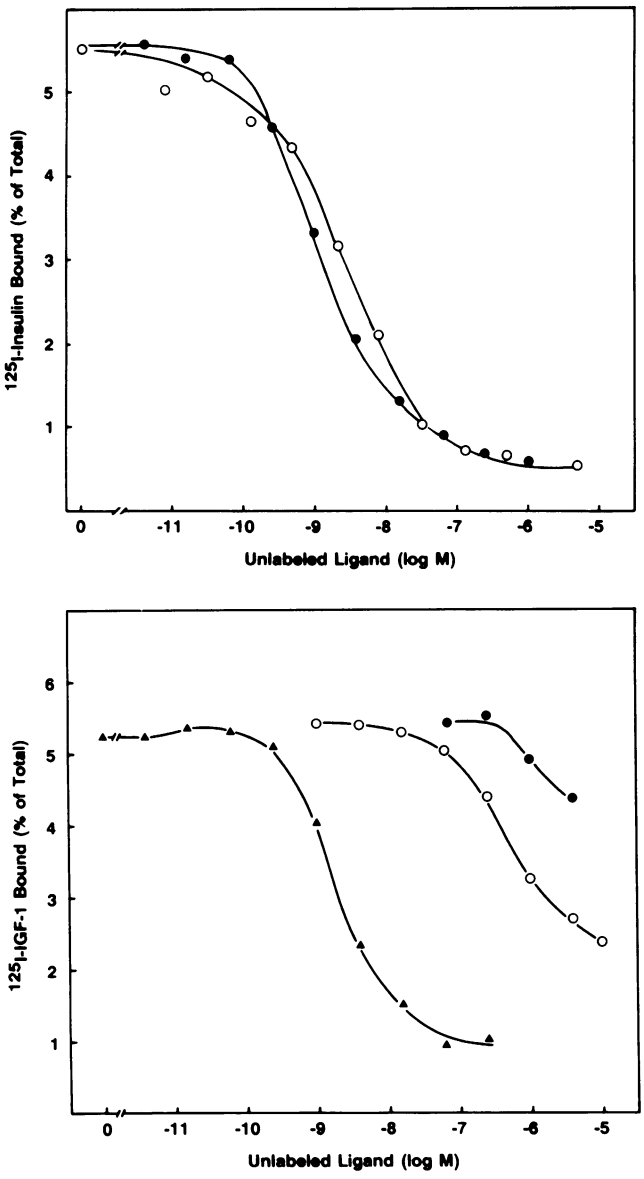

FIG. 2. Binding of B29,B29′-suberoyl-insulin to insulin and IGF-I receptors in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were incubated for 17 hr at 4°C in the presence of ^{125}I-insulin (*Upper*) or ^{125}I-IGF-I (*Lower*) and the indicated concentrations of unlabeled insulin (○), B29,B29′-suberoyl-insulin (●), or IGF-I (▲). Data represent means of triplicate samples from a representative experiment.

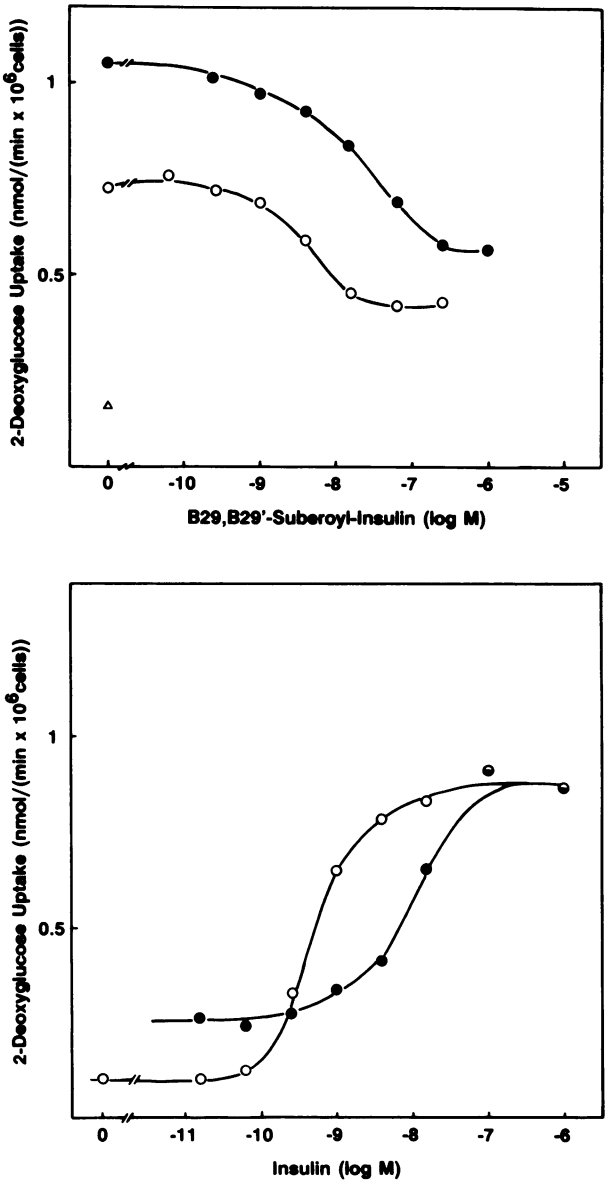

FIG. 3. Inhibitory effect of B29,B29′-suberoyl-insulin on insulin-stimulated glucose transport activity in 3T3-L1 adipocytes. (*Upper*) Glucose transport was assayed as described in the legend of Fig. 1 after incubation of differentiated 3T3-L1 adipocytes for 15 min in the presence of 1 nM (○) or 10 nM (●) insulin and the indicated concentrations of B29,B29′-suberoyl-insulin. △, Basal glucose transport in the absence of insulin and B29,B29′-suberoyl-insulin. (*Lower*) Differentiated 3T3-L1 adipocytes were incubated for 2 hr in serum-free medium, washed with Krebs–Ringer Hepes buffer, and exposed to the indicated concentrations of insulin (○) or insulin plus 250 nM B29,B29′-suberoyl-insulin (●) in Krebs–Ringer Hepes buffer for 15 min. Thereafter, 2-deoxyglucose uptake was assayed as described.

On the basis of the discrepancy between efficacy and binding affinity of B29,B29′-suberoyl-insulin (Figs. 1 and 2), we anticipated the derivative to act as an antagonist of insulin-stimulated glucose transport and DNA synthesis. Indeed, when cells were exposed to a combination of insulin and B29,B29′-suberoyl-insulin, a concentration-dependent inhibition of 2-deoxyglucose uptake (Fig. 3 *Upper*) or thymidine incorporation (Fig. 4 *Upper*) was observed. Half maximally inhibiting concentrations of the dimer were higher at the higher insulin concentrations (Figs. 3 and 4 *Upper*), indicating that the inhibitory potency of the dimer depended on the insulin concentration. Accordingly, as Figs. 3 and 4 *Lower* illustrate, the dimer shifted the concentration–response curves of insulin action on glucose transport and DNA synthesis to higher insulin concentrations; the maximum effect of insulin was not affected. These data are in agreement with the assumption of a competitive type of inhibition. The moderate increase in glucose transport activity or DNA synthesis produced by B29,B29′-suberoyl-insulin in the presence of substimulatory insulin concentrations (10^{-10} M) is due to the weak agonist action (intrinsic activity) of the dimer (Figs. 3 and 4 *Lower*).

Similarly, the B29,B29′-suberoyl-insulin inhibited the insulin-stimulated autophosphorylation of the receptor β-subunit from 3T3-L1 cells by approximately 50% (Fig. 5). The effects of the dimerized derivative were investigated at two insulin concentrations. As in the experiments shown in Figs. 3 and 4, the inhibitory potency of the dimer was found to be higher at the lower insulin concentration (Fig. 5). In the absence of insulin, the dimer (10^{-6} M) stimulated receptor autophosphorylation to a lesser extent than insulin, thus acting as a partial agonist (data not shown).

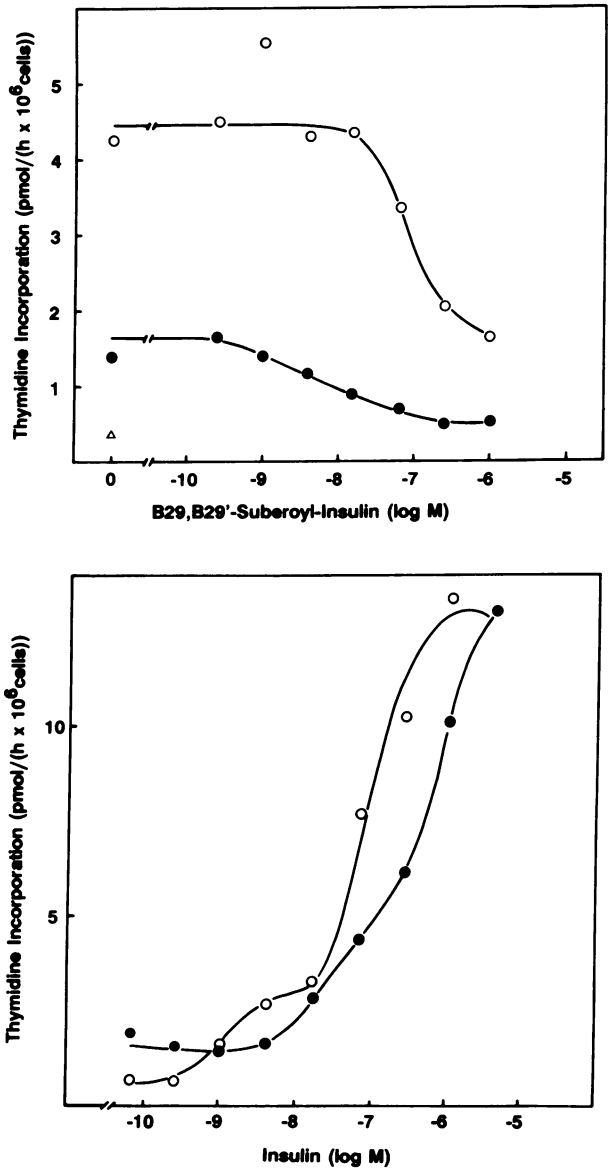

FIG. 4. Inhibitory effect of B29,B29′-suberoyl-insulin on insulin-stimulated DNA synthesis in 3T3-L1 adipocytes. (*Upper*) Thymidine incorporation into DNA was assayed as described in the legend of Fig. 1 after incubation of cells for 17.5 hr in the presence of 3.9 nM (●) or 100 nM (○) insulin and the indicated concentrations of B29,B29′-suberoyl-insulin. △, Basal thymidine incorporation in the absence of added hormone. The figure shows means of triplicate samples from a representative experiment. (*Lower*) Differentiated 3T3-L1 adipocytes were cultured in serum-free medium for 48 hr and were exposed to the indicated concentrations of insulin (○) or insulin plus 250 nM B29,B29′-suberoyl-insulin (●) for 17.5 hr. Thereafter the cells were incubated with [^{3}H]thymidine for 120 min, and incorporation of tritium into total DNA was assayed. The figure shows means of triplicate samples from a representative experiment.

In striking contrast to the effect of the dimer on glucose transport in the 3T3-L1 adipocyte, B29,B29′-suberoyl-insulin had been reported to produce a full insulin-like stimulation of glucose transport activity in the mature rat fat cell (4, 5). In experiments performed in parallel with the series depicted in Fig. 1, B29,B29′-suberoyl-insulin (10^{-7} M) elicited the same stimulation of 3-*O*-methylglucose uptake in rat fat cells as a maximally stimulating concentration of insulin did: 447.5 ± 50.2 fmol/(cell·μl of lipid) as compared to 443.4 ± 55.1 in the presence of insulin (10^{-8} M); the basal rate was 62.6 ± 7.1 fmol/(cell·μl of lipid) (mean ± SEM of three separate experiments). In addition, the dimer also elicited the maximal insulin response, when glucose transport activity in rat fat cells was assayed with the 2-deoxyglucose uptake assay: 12.8 ± 3.1 vs. 13.2 ± 2.8 pmol/(min·μl of lipid) in the presence of insulin; basal rates were 3.4 pmol/(min·μl of lipid).

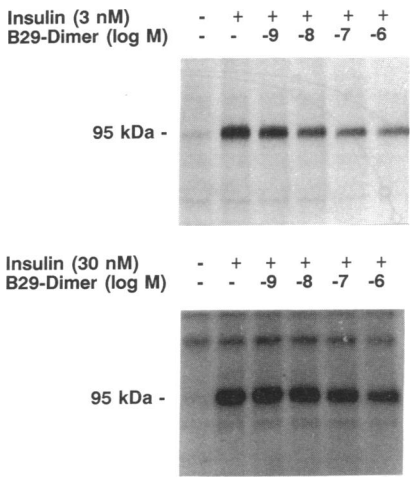

FIG. 5. Inhibitory effect of B29,B29′-suberoyl-insulin on insulin-stimulated autophosphorylation of 95-kDa insulin receptor β-subunit from 3T3-L1 cells. Insulin receptor was partially purified and incubated in the presence (+) or absence (−) of insulin (*Upper*, 3 nM; *Lower*, 30 nM) with or without (−) the indicated concentrations (log M) of B29,B29′-suberoyl-insulin for 20 min. Autophosphorylation was initiated by addition of labeled ATP and was terminated after 10 min. Samples were separated by electrophoresis, and phosphate incorporation was detected by autoradiography.

To further establish that the dimer is a pure insulin receptor agonist in mature rat adipocytes, we studied its effects on glucose transport in combination with different concentrations of insulin. As is illustrated in Fig. 6, the three concentrations of the dimer employed (0.25, 0.8, 2.5 nM) enhanced the effect of insulin on glucose transport; no antagonistic effect of the dimer was observed at any concentration of insulin.

DISCUSSION

The present data indicate that the covalently dimerized insulin derivative B29,B29′-suberoyl-insulin is an antagonist of insulin's effects with a moderate intrinsic activity. We know of no other inhibitor that antagonizes insulin action at the receptor level. The dimer possesses a moderate intrinsic activity which limits its inhibitory efficacy, but it is conceivable that derivatives with lower intrinsic activity and higher binding affinity can be developed in the future from this lead substance. Specific receptor antagonists can be valuable tools to define the molecular basis of ligand–receptor interaction and to distinguish different receptors or pathways of action. Along these lines, the dimerized insulin derivative revealed a striking difference of glucose transport stimulation in rat adipocytes and the 3T3-L1 adipocyte.

Surprisingly, the present data differentiated glucose transport activation in the mature adipocyte from that in the 3T3-L1 adipocyte in that the former could not be antagonized by the dimer. Two reasons for the striking difference in glucose transport stimulation in the two cell types can be discussed. First, in mature rat fat cells occupation of as little as 5% of the total receptors suffices to produce full glucose transport stimulation (15). The remaining 95% of the receptors are called "spare receptors" and are assumed to be identical to those mediating the response to the hormone. In contrast to rat adipocytes, 3T3-L1 adipocytes appear to be devoid of spare receptors, since glucose transport stimulation

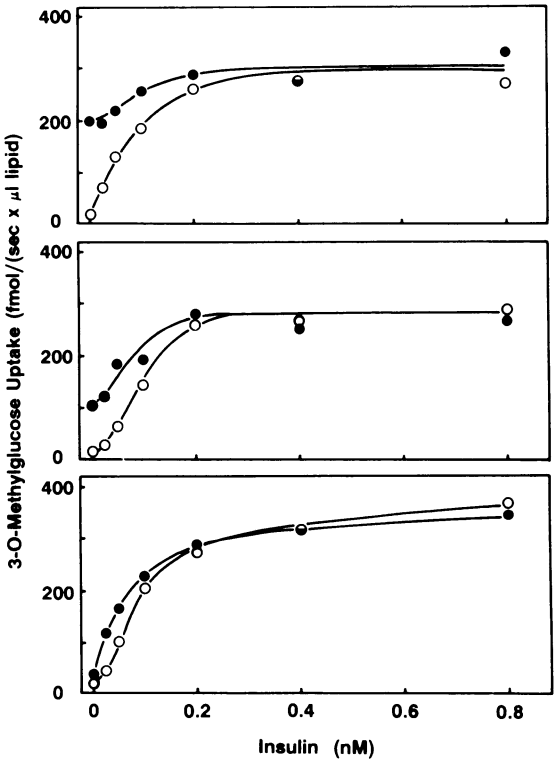

FIG. 6. Additive effect of B29,B29′-suberoyl-insulin on insulin-stimulated glucose transport in isolated rat adipocytes. Rat fat cells were isolated and were incubated with a combination of the indicated concentrations of insulin and 0.25 nM (*Bottom*), 0.8 nM (*Middle*), or 2.5 nM (*Top*) B29,B29′-suberoyl-insulin at 37°C for 20 min, and glucose transport activity was assayed. ○, Control experiments in which cells were treated with insulin only. Data are means of duplicate samples from a representative experiment.

and kinase activation have essentially the same EC_{50} (Figs. 1 and 2 and ref. 16). Based on the assumption that binding to the spare receptors can compensate for the lower intrinsic activity of B29,B29′-suberoyl-insulin, it is conceivable that the maximum stimulation of glucose transport can be achieved with the dimer in adipocytes. Consequently, in spite of its low intrinsic activity, the dimer would fully stimulate glucose transport in cells equipped with spare insulin receptors, and it would antagonize insulin action in cells if spare receptors are absent. Thus, based on the assumptions outlined above, the existence of spare receptors in the rat adipocyte might account for the full agonist effect of the dimer in this cell.

As a second explanation for the diverse effects of the derivative, heterogeneity of insulin receptors in the two cell types has to be considered. The analysis of the concentration–response curves of glucose transport stimulation supports the notion of a functional difference between insulin receptors in 3T3-L1 cells and the rat fat cell. In 3T3-L1 cells, the concentration–response curves of transport stimulation by insulin follow receptor occupancy according to single-site binding kinetics (16) with a Hill coefficient near 1. In contrast, in the mature adipocyte the concentration–response curve extends over less than two orders of magnitude (Hill coefficient <1) (13–15), indicating the presence of a mechanism amplifying the effect of insulin at low receptor occupation. Further, the absolute number of insulin receptors per cell (approximately 10^5 receptors per cell) is roughly equal in both cell types (17). Thus, the presence of spare receptors cannot simply be due to an excess of receptors, but might rather reflect a qualitative difference. It cannot be excluded, therefore, that the dimerized insulin derivative distinguishes two functionally different forms of the insulin receptor present in differentiated 3T3-L1 cells and rat adipocytes.

In 3T3-L1 adipocytes, the dimer competitively antagonized insulin action on both glucose transport and DNA synthesis. The stimulatory effect of insulin on DNA synthesis appeared to consist of two components, the major portion of which was produced by insulin concentrations exceeding those necessary for receptor occupation. This component might thus be mediated by other receptors–e.g., IGF-I receptors. The low-EC_{50} component, however, corresponded to the insulin concentrations stimulating glucose transport as well as to the binding curves. Thus, it is reasonable to conclude that at least a portion of the effect of insulin is mediated by its own receptor. Since this component was also inhibited by B29,B29′-suberoyl-insulin, it can be concluded that the dimer inhibits both glucose transport and DNA synthesis by binding to the insulin receptor. Thus, the insulin receptor antagonist failed to differentiate rapid metabolic effects and long-term actions of insulin. Consequently, the present data do not contradict the view that insulin can stimulate both glucose transport and mitogenesis via the same receptor, and probably via the same initial intracellular signal.

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