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Monoclonal antibodies to the insulin receptor mimic metabolic effects of insulin but do not stimulate receptor autophosphorylation in transfected NIH 3T3 fibroblasts

(glucose uptake/thymidine uptake/tyrosine kinase)

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ABSTRACT **The metabolic actions of insulin and anti-insulin receptor monoclonal antibodies were compared with their effects on insulin receptor phosphorylation in mouse NIH 3T3 fibroblasts transfected with human insulin receptor cDNA. In serum-starved NIH 3T3 HIR3.5 cells, uptake of 2-deoxy-[^{3}H]glucose was stimulated up to 2-fold after 30 min with insulin, with a half-maximal effect at 0.1 nM insulin. Incorporation of [^{3}H]thymidine was stimulated ≈12-fold after a 16-hr preincubation with insulin, with a half-maximal effect at 2 nM insulin. Phosphorylation of insulin receptor β-subunit in cells prelabeled with [^{32}P]phosphate was increased 10- to 20-fold within 5 min of adding insulin, with a half-maximal effect at ≈3 nM insulin. Monoclonal antibodies reacting with four different epitopes on the insulin receptor mimicked the effect of insulin on 2-deoxyglucose uptake. These antibodies also stimulated thymidine incorporation, although the maximum stimulation was only ≈30% that of insulin. Two antibodies (25-49 and 83-14) showed a similar concentration dependence to insulin in their metabolic effects and in the inhibition of ^{125}I-labeled insulin binding to cells. The other two antibodies (83-7 and 18-44) were somewhat less potent and did not inhibit insulin binding. None of the antibodies significantly increased insulin receptor phosphorylation at concentrations up to 100 nM, which at least in the case of 25-49 and 83-14 was sufficient for full receptor occupancy. It is concluded that the insulin-like metabolic effects of antibodies involve a mechanism of receptor activation that is independent of autophosphorylation and hence that receptor autophosphorylation is not an essential step in triggering at least some events in the insulin signaling pathway.**

The molecular mechanism whereby insulin regulates a diverse array of metabolic processes is not fully understood (1, 2). The insulin receptor, which is a heterotetrameric membrane glycoprotein (3), possesses tyrosine-specific protein kinase activity in the cytosolic domain of its β-subunit (4). Substantial evidence implicates activation of this tyrosine kinase by insulin as an essential early step in the intracellular signaling pathway (2–7), although the nature of its involvement is unclear. Various potential substrates have been described, either as unidentified proteins that are phosphorylated on tyrosine in response to insulin in intact cells (8–10), or as components of established signaling pathways that are phosphorylated by insulin receptors *in vitro* (11, 12). Alternatively, the important reaction may be phosphorylation of the receptor itself (13, 14), which would be expected to alter the conformation of the cytosolic domain and its interaction with other cellular proteins. This autophosphorylation also increases the kinase activity toward exogenous substrates (14, 15).

Anti-receptor antibodies, both polyclonal and monoclonal, have been useful probes of insulin receptor structure and function, and at least some of these are insulin mimetic (16–18). However, there has been confusion over whether or not polyclonal antibodies necessarily activate the receptor kinase (19, 20). We have obtained monoclonal antibodies to a number of distinct epitopes on the insulin receptor (21), which mimic metabolic effects of insulin in adipocytes (22) and in some but not all cases stimulate the kinase activity of solubilized receptor (23). The present study was undertaken to clarify the role of receptor kinase in mediating insulin-like effects, making use of metabolically responsive mouse NIH 3T3 cells that have been transfected with insulin receptor cDNA (24).

MATERIALS AND METHODS

General. Bovine insulin, 2-deoxyglucose, aprotinin, phloretin, *N*-ethylmaleimide, and bovine serum albumin were from Sigma. Tissue culture media were from GIBCO, and fetal calf serum was from various suppliers. Radiochemicals were purchased from Amersham, except 2-deoxy-D-[1,2-^{3}H]glucose, which was from NEN. Mono-[^{125}I]iodoinsulin (^{125}I-insulin) (specific activity, 150–200 μCi/μg; 1 Ci = 37 GBq) was prepared as described (25).

Monoclonal antibodies to human insulin receptors were as described (21) and were purified from ascites fluids by precipitation with $(NH_4)_2SO_4$ followed by chromatography on hydroxylapatite (26). Purity was at least 95% as estimated by scanning of $NaDodSO_4$/polyacrylamide gels. Monovalent Fab fragments of antibodies were obtained by digestion with pepsin and reduction with cysteine (27) and were purified by gel filtration.

NIH 3T3 HIR3.5 cells (24) were cultured in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum for 2–3 days after passage, until near confluence, before experiments.

Insulin Binding Assays. Cells on 24-well plates were incubated with insulin or antibody in 0.25 ml of Hepes-buffered DMEM (25 mM Hepes/7 mM bicarbonate, pH 7.4) containing 1 mg of bovine serum albumin per ml, 1 mM *N*-ethylmaleimide and 250 kallikrein inhibitor units of aprotinin per ml for 30 min at 4°C, before addition of 0.05 ml of ^{125}I-insulin (≈30,000 dpm; final concentration, ≈0.1 nM). After 4 hr, cells were washed with ice-cold phosphate-

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buffered saline (PBS) and solubilized with 0.03% $NaDodSO_4$. Nonspecific binding was determined in the presence of 1 μM insulin and was normally ≈0.1% of total radioactivity.

2-Deoxyglucose Uptake. Cells on 24-well plates were serum-starved by incubation for 16 hr in Hepes-buffered DMEM containing 1 mg of bovine serum albumin per ml, and then incubated for 30 min at 37°C with insulin or antibody in 0.4 ml of the same medium without glucose, before addition of 0.1 ml of 2-deoxy-D-[1,2-^{3}H(N)]glucose (0.4 μCi; final concentration, 0.1 mM). After 10 min, cells were washed with ice-cold PBS containing 0.3 mM phloretin and then solubilized with 0.03% $NaDodSO_4$.

Thymidine Uptake. Cells on 24-well plates were incubated for 24 hr in DMEM containing 0.5% fetal calf serum, and then for 16 hr at 37°C with insulin or antibody in 0.9 ml of Hepes-buffered DMEM containing 1 mg of bovine serum albumin per ml, before addition of 0.1 ml of [6-^{3}H]thymidine (0.5 μCi; 22 Ci/mmol). After 2 hr, cells were washed with ice-cold PBS and solubilized with 0.03% $NaDodSO_4$. Trichloroacetic acid was added to a final concentration of 10% (wt/vol) and the precipitate was collected by filtration onto glass fiber filters (Whatman), which were washed with 4 × 5 ml of 10% trichloroacetic acid before drying.

Insulin Receptor Phosphorylation. Cells on 6-well plates were incubated for 16 hr in Hepes-buffered DMEM containing 1 mg of bovine serum albumin per ml. This was then replaced with 0.7 ml of the same medium (25 mM glucose) or, in some experiments, medium without glucose, and 0.05 ml of [^{32}P]phosphate (≈200 μCi, carrier-free) was added to each well. After 4 hr at 37°C, insulin or antibody was added, and incubations continued until termination by aspirating the medium and freezing the cells in liquid N_2. Cells were solubilized and receptors were immunoprecipitated with anti-insulin receptor antibody (either rabbit serum IR-1 or mouse ascites 83-14) as described (28). The washed immunoprecipitates were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography (28, 29).

RESULTS

Effects of Antibodies on Insulin Binding. To establish that glycosylation differences between receptor expressed in NIH 3T3 HIR3.5 cells and in normal human tissues (24) did not affect reactivity with monoclonal antibodies, the effects of antibodies on insulin binding were investigated (Fig. 1). Antibodies used were representative of those described (21): 25-49, 83-14, and 47-9 react with sites on the α-subunit; 18-44 reacts with the β-subunit; and 83-7 reacts with a site clearly distinct from all other antibodies, which has not been localized to either subunit. None of the antibodies reacts with mouse receptors. Antibodies previously shown to inhibit ^{125}I-insulin binding to human adipocytes and IM-9 cells (21, 22) similarly inhibited binding to NIH 3T3 HIR3.5 cells. Half-maximal inhibition of ^{125}I-insulin binding was achieved with 2–3 nM insulin or antibodies 25-49, 83-14, and 47-9. Scatchard analysis of the binding data suggested that there were ≈1 × 10^6 high-affinity receptors per cell, with K_d = 1 nM. Monovalent Fab fragments behaved similarly to bivalent antibodies although with slightly lower binding affinities. Half-maximal inhibitory concentrations for Fab fragments were ≈2 nM (83-14), 5 nM (25-49), and 7 nM (47-9) (data not shown).

Antibodies 83-7 and 18-44, which do not inhibit insulin binding to human cells, failed also to inhibit binding to NIH 3T3 HIR3.5 cells (Fig. 1). Nevertheless, these antibodies did react with the human receptor expressed in mouse cells, with affinities comparable to the other antibodies, as demonstrated by binding studies with ^{125}I-labeled antibodies (K.S. and M.A.S., unpublished data).

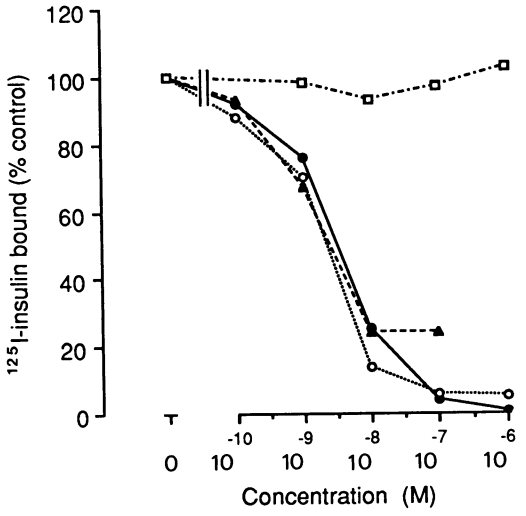

FIG. 1. Effect of antibodies on insulin binding. Binding of ^{125}I-insulin was determined in the presence of unlabeled insulin (●; mean of eight experiments) or monoclonal antibodies 25-49 (○; mean of two experiments), 83-14 (▲; mean of two experiments), and 83-7 (□; one experiment). Measurements were performed in triplicate within each experiment and results are expressed as a percentage of ^{125}I-insulin bound in the absence of unlabeled insulin or antibody (typically 5000–10,000 dpm).

Effect of Antibodies and Insulin on Uptake of 2-Deoxyglucose. We tested the effect of insulin and antibodies on the uptake of the nonmetabolizable sugar 2-deoxyglucose in serum-starved cells. The maximum stimulation of uptake by insulin varied from 1.5- to 2-fold in different experiments and the mean half-maximally effective insulin concentration was 0.1 nM (Fig. 2).

Monoclonal antibodies 25-49 and 83-14 stimulated 2-deoxyglucose uptake to ≈90% of the maximal insulin effect at 100 nM, with half-maximal effects at ≈0.4 and ≈0.8 nM, respectively (Fig. 2). Antibody 83-7 also produced a substantial stimulation but was rather less potent (half-maximal at ≈12 nM), while 18-44 produced only a submaximal (35%) stimulation even at 100 nM (data not shown).

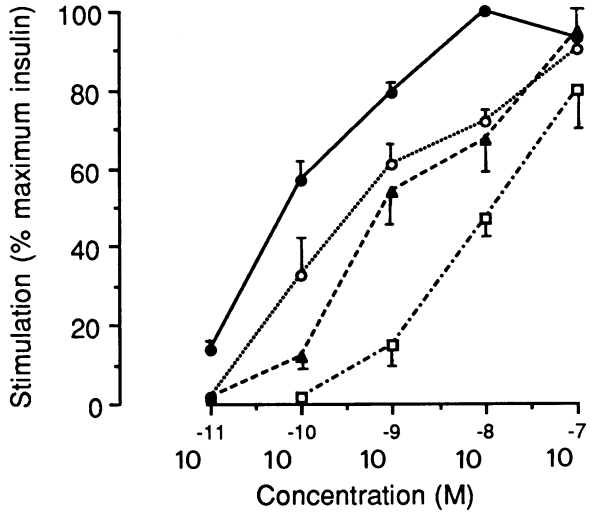

FIG. 2. Effect of antibodies on deoxyglucose uptake. Stimulation of 2-deoxy[^{3}H]glucose uptake was determined in the presence of insulin (●) and monoclonal antibodies 25-49 (○), 83-14 (▲), and 83-7 (□). The results, expressed as a percentage of the maximum stimulation by 100 nM insulin within each experiment, are the mean ± SEM of three (83-14, 83-7) or four (insulin, 25-49) independent experiments, with triplicate determinations within experiments.

Antibody 47-9, which inhibits rather than mimics insulin action in adipocytes (22), had no effect on the basal uptake of 2-deoxyglucose at concentrations up to 100 nM (data not shown). Control antibodies of irrelevant specificity were similarly without effect. Monovalent Fab fragments of antibodies 25-49, 83-14, and 83-7 had no effect at concentrations up to 1 nM (data not shown) and a small stimulation (≈20%) at higher concentrations was consistent with ≈1% contamination with bivalent antibody.

Effects of Antibodies and Insulin on Thymidine Uptake. Serum-starved NIH 3T3 HIR3.5 cells showed a dramatic stimulation by insulin of thymidine incorporation into trichloroacetic acid-precipitable material, which varied from 5- to 20-fold in different experiments (mean, 12-fold), although absolute levels of stimulated incorporation were similar in all experiments. A half-maximal effect was obtained at 2 nM insulin (Fig. 3). The apparently lower sensitivity to insulin of thymidine incorporation compared to 2-deoxyglucose uptake may reflect insulin degradation during the longer preincubation period.

Antibodies 25-49, 83-14, and 83-7 consistently stimulated thymidine uptake, but to a lower maximum than insulin (Fig. 3). The half-maximally effective antibody concentrations were ≈0.2 nM (25-49), ≈0.5 nM (83-14), and ≈2 nM (83-7), somewhat lower than the corresponding values for stimulation of 2-deoxyglucose uptake. Antibody 18-44 reacted with a potency similar to 83-7 (data not shown). Antibody 47-9 was without effect on thymidine uptake at concentrations up to 100 nM (data not shown), and control monoclonal antibodies of irrelevant specificity were similarly ineffective. Monovalent Fab fragments of all the antibodies were also without effect at a concentration of 10 nM (data not shown).

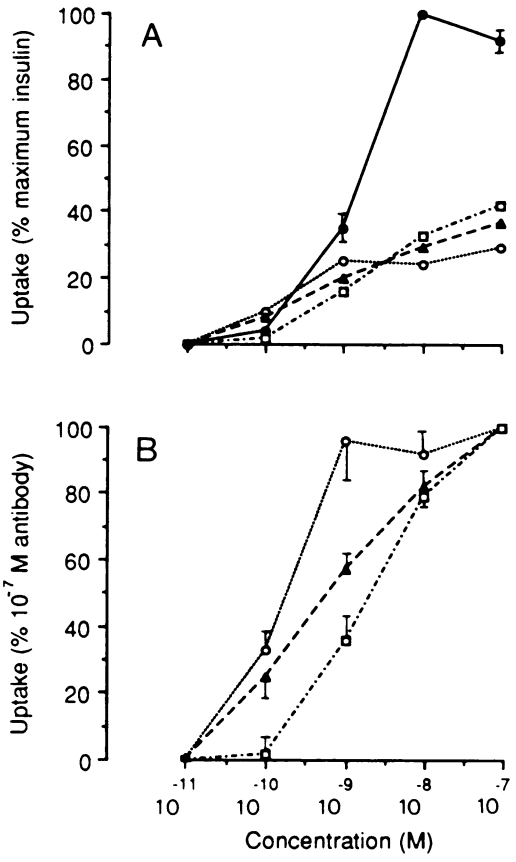

FIG. 3. Effect of antibodies on thymidine uptake. Stimulation of [^{3}H]thymidine uptake into trichloroacetic acid-precipitable material was determined in the presence of insulin (●) and monoclonal antibodies 25-49 (○), 83-14 (▲), and 83-7 (□). Measurements were performed in triplicate within each experiment. (*A*) Results, expressed as a percentage of the maximum stimulation by insulin within each experiment, are the means ± SEM of 15 experiments for insulin and the means of three experiments for each of the antibodies. (*B*) Results, expressed as a percentage of the maximum stimulation by each antibody (for the same data as in *A*), are the means ± SEM of three independent experiments.

The difference in maximal effects of insulin and antibodies is probably related to the much more potent down-regulation of insulin receptors by antibodies (30) during the 16-hr preincubation period before measurement of thymidine uptake. When cells were preincubated with insulin (100 nM) plus antibody (100 nM), the stimulation of thymidine uptake was only at the submaximal level expected for antibody alone, compared to the maximal effect of insulin alone (data not shown). This was the case even with antibody 83-7, which does not directly inhibit insulin binding, as well as with 25-49 and 83-14, which competitively inhibit binding in addition to down-regulating receptors.

Effect of Insulin and Antibodies on Receptor Autophosphorylation. Insulin rapidly stimulated the phosphorylation of its receptor in intact NIH 3T3 HIR3.5 cells, predominantly on tyrosine residues (28), reaching a maximum increase of ≈10-fold within 5 min, which was maintained for 1 hr (Fig. 4). However, there was no increase in receptor phosphorylation in the presence of antibody 25-49 at a concentration (100 nM) sufficient for full receptor occupancy (see Fig. 1), either after short incubation times (Fig. 4) or after 30–60 min (data not shown). When phosphorylation was assessed in glucose-free medium, again no effect of antibody 25-49 was observed, although in the same experiment cells treated identically showed stimulation of 2-deoxyglucose uptake by the antibody. When labeling was carried out in phosphate-free medium (28, 31) to increase the level of radioactive incorporation into the receptor, again no effect of antibody was detected.

In experiments with solubilized placental receptor, antibodies stimulated receptor phosphorylation only within a narrow concentration range (23). However, antibody 25-49, tested over a wide concentration range, produced no significant increase in receptor phosphorylation in intact cells (Fig. 5). Antibody 83-14 at the highest concentration tested (100 nM) produced an ≈2-fold increase in receptor phosphorylation in the experiment shown, an effect equivalent to that induced by ≈0.1 nM insulin (Fig. 5). However, this was not reproducibly observed in other experiments. Antibodies 83-7 and 18-44 were also ineffective at concentrations up to 1 μM (data not shown).

The effect of insulin on receptor phosphorylation was clearly antagonized by antibodies 25-49 and 47-9 (Fig. 5) over a similar concentration range to that in which they inhibited insulin binding (Fig. 1). These antibodies appeared very similar in their effects on receptor phosphorylation, although 25-49 mimics and 47-9 inhibits all the metabolic effects of insulin tested to date. Antibody 83-7, which does not inhibit insulin binding, did not antagonize insulin-stimulated receptor phosphorylation (Fig. 6), thus ruling out the possibility

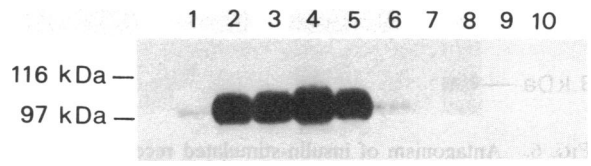

FIG. 4. Time course of insulin receptor autophosphorylation. Cells prelabeled with [^{32}P]phosphate were incubated with 10^{-7} M insulin or antibody before solubilization, immunoprecipitation, analysis on a 10% polyacrylamide gel, and autoradiography. Times of incubation were 0 (lanes 1 and 6), 1 min (lanes 2 and 7), 2 min (lanes 3 and 8), 5 min (lanes 4 and 9), and 10 min (lanes 5 and 10), with insulin (lanes 2–5) or antibody 25-49 (lanes 7–10).

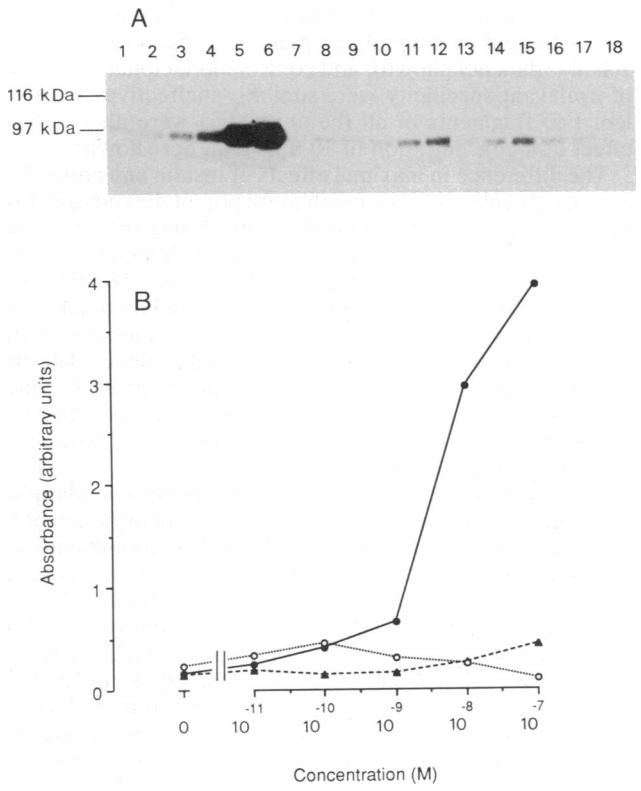

FIG. 5. Concentration dependence of insulin receptor phosphorylation. (*A*) Cells prelabeled with [^{32}P]phosphate were incubated for 5 min with insulin or antibodies, before solubilization, immunoprecipitation, analysis on a 10% polyacrylamide gel, and autoradiography. Cell incubations contained no additions (lanes 1, 7, and 13), insulin (lanes 2–6), antibody 83-14 (lanes 8–12), or antibody 25-49 (lanes 14–18) at concentrations 10^{-11} M (lanes 2, 8, and 14), 10^{-10} M (lanes 3, 9, and 15), 10^{-9} M (lanes 4, 10, and 16), 10^{-8} M (lanes 5, 11, and 15), or 10^{-7} M (lanes 6, 12, and 18). (*B*) Phosphorylation of insulin receptor β-subunit (97 kDa) was quantified by densitometric scanning of the autoradiogram shown in *A*. The results, expressed in arbitrary units, are for insulin (●), antibody 25-49 (○), and antibody 83-14 (▲).

that the lack of stimulation of receptor phosphorylation by antibodies was due to a simultaneous activation of protein phosphatase.

DISCUSSION

The insulin-like effects of polyclonal (16, 17) and monoclonal (18, 22) anti-insulin receptor antibodies are well documented and the mechanism of these effects has been studied in an attempt to gain insights into the action of insulin itself. To avoid problems of interpretation inherent in the use of polyclonal antisera (19, 20, 32–34), which may contain a mixture of antibodies with differing activities and whose concentrations are difficult to quantify, we have investigated the effects of monoclonal anti-insulin receptor antibodies. In previous studies, we showed that antibodies reacting with several distinct epitopes, both close to and distant from the insulin-binding site, elicited insulin-like metabolic effects in adipocytes (22). Most of these antibodies also stimulated the kinase activity of solubilized placental receptor, although one of them (antibody 25-49) conspicuously failed to do so (23). A similar discrepancy has been reported for monoclonal antibodies MA-5 and MA-20 (18). We therefore considered it important to examine the relationship between metabolic effects and kinase activity within metabolically responsive intact cells. We used the cell line NIH 3T3 HIR3.5 in which transfection with human insulin receptor cDNA results in high level expression of insulin receptors, conferring insulin sensitivity on cells that are otherwise unresponsive (24). The half-maximal insulin concentration for acute metabolic stimulation (0.1 nM; Fig. 2) was ≈30 times lower than that for receptor binding (3 nM; Fig. 1) and stimulation of receptor phosphorylation (≈3 nM; Fig. 5), although comparison is complicated by differences in temperature and incubation time for the different experiments.

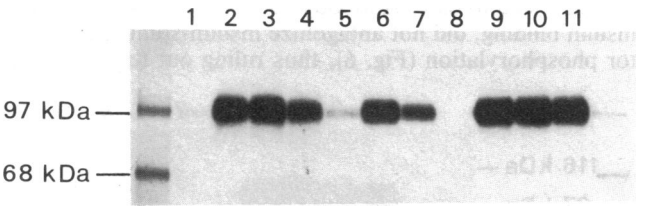

FIG. 6. Antagonism of insulin-stimulated receptor phosphorylation by antibodies. Cells prelabeled with [^{32}P]phosphate were incubated for 5 min with insulin and antibodies, before solubilization, immunoprecipitation, analysis on a 10% polyacrylamide gel, and autoradiography. Cell incubations contained no additions (lane 1) or 10^{-7} M insulin (lanes 2–11), together with antibody 47-9 (lanes 3–5), antibody 25-49 (lanes 6–8), or antibody 83-7 (lanes 9–11) at concentrations of 10^{-9} M (lanes 3, 6, and 9), 10^{-8} M (lanes 4, 7, and 10), or 10^{-7} M (lanes 5, 8, and 11).

Several antibodies (25-49, 83-14, 18-44, 83-7) elicited both short- and long-term insulin-like effects in NIH 3T3 HIR3.5 cells, stimulating uptake of 2-deoxyglucose and thymidine (Figs. 2 and 3). As in adipocytes (22), antibodies 25-49 and 83-14 had similar affinity to insulin in binding to receptor (Fig. 1) and were also comparable in their metabolic potency (Figs. 2 and 3), indicating a similar efficiency of coupling of insulin- and antibody-occupied receptors to the signaling pathway responsible for metabolic effects. Antibodies 83-7 and 18-44 were less potent, but whether this reflects lower binding affinities, or less efficient coupling to subsequent signaling steps, is at present unclear.

None of the antibodies tested significantly increased receptor phosphorylation in intact cells at concentrations up to 100 times those necessary for maximum metabolic stimulation and sufficient for full receptor occupancy (Figs. 4 and 5). Insulin under the same conditions markedly increased phosphorylation. Antibodies 83-14 and 25-49, which inhibit insulin binding and insulin-induced receptor phosphorylation (Figs. 1 and 6), are therefore very different from insulin in the relationship between receptor occupancy and stimulation of autophosphorylation, although similar to insulin in terms of the occupancy–metabolic response relationship. Metabolic effects of antibodies clearly occur in the absence of any detectable increase in autophosphorylation of receptor *in situ*.

These results were unexpected in view of previous work showing that some of these antibodies stimulate autophosphorylation of solubilized placental receptor (23) and solubilized receptor from NIH 3T3 HIR3.5 cells (R.M.O., J. Tavare, and K.S., unpublished data). It may be that steric constraints on receptor *in situ* limit the conformational changes that take place on antibody binding, so that autophosphorylation no longer occurs. Both kinase activation in soluble preparations and metabolic effects in intact cells require antibody bivalency, as previously shown for polyclonal antisera (18). Both inter- and intramolecular cross-linking of solubilized receptors occurs, although only the latter appears to result in kinase activation (23). The high receptor density on NIH 3T3 HIR3.5 cells may favor intermolecular cross-linking that does not stimulate the kinase but nevertheless results in some other type of receptor activation.

A second anomaly arises from the previous demonstration that mutant receptors lacking kinase activity, when trans-

fected into CHO cells, were unable to mediate insulin-like effects of antibodies 25-49, 83-14, and 18-44 (5). It was not shown in those experiments whether antibodies induced phosphorylation in cells expressing wild-type receptor (CHO-IR.lys). However, in CHO-T cells (31), which express a level of receptors comparable to NIH 3T3 HIR3.5 cells and higher than CHO-IR.lys cells, we again could see no increase in receptor phosphorylation in response to any of the monoclonal antibodies tested in the present study (35). We have recently shown, by immunoblot analysis of extracts from NIH 3T3 HIR3.5 cells with anti-phosphotyrosine antibody, that the anti-receptor monoclonal antibodies fail to induce rapid phosphorylation not only of the receptor itself, but also of an endogenous substrate of ≈180 kDa although a small increase was detectable after 30 min (N.P.J.B. and K.S., unpublished work). This unidentified protein shows a marked and rapid increase in phosphorylation in response to insulin in these as in other cells (8). Paradoxically, therefore, the insulin-like effects of antibodies appear to require receptor with an intact ATP-binding site and kinase activity but do not involve the activation of this kinase.

The mechanisms underlying the metabolic effects of anti-receptor antibodies therefore remain unclear, and the possibility cannot be ruled out that these are distinct from those responsible for insulin action. Thus, the marked internalization of receptor induced by antibodies (30) could result in delivery of basally active kinase to sensitive intracellular sites or in some way trigger reciprocal movement of subcellular vesicles and glucose transporters to the cell surface. Alternatively, it may be that antibodies, by cross-linking, induce a receptor conformation that mimics the autophosphorylated state in allowing a nonenzymatic interaction with other regulatory components such as G proteins, serine-specific protein kinases, or phospholipases (2). If so, the failure of mutant receptors to respond to antibody indicates that the effects of amino acid substitution at the ATP-binding site are not confined to inhibition of receptor kinase activity, casting doubt on the validity of experiments with such mutants as evidence for the role of kinase activity in the actions of insulin itself.

Note Added in Proof. After submission of this manuscript, Hawley *et al.* (36) similarly reported insulin-like metabolic effects of monoclonal anti-receptor antibodies in the absence of receptor autophosphorylation in transfected rat HTC cells expressing human insulin receptors.

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