

Diabetes

JOURNAL OF
THE AMERICAN
DIABETES
ASSOCIATION

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ABSTRACT FORM FOR 54TH SCIENTIFIC SESSIONS

The Sulfonylurea Drug, Glimepiride, Stimulates Glucose Transport, Glucose Transporter Translocation, and Dephosphorylation In Insulin-Resistant Rat Adipocytes In Vitro

GÜNTER MÜLLER AND SUSANNE WIED

Sulfonylurea drugs are widely used in the therapy of NIDDM. The improvement of glucose tolerance after long-term treatment of NIDDM patients with the drug can be explained by stimulation of glucose utilization in peripheral tissues that are characterized by insulin resistance in these patients. We studied whether the novel sulfonylurea drug, glimepiride, stimulates glucose transport into isolated insulin-resistant rat adipocytes. After long-term incubation of the cells in primary culture with high concentrations of glucose, glutamine, and insulin, stimulation of glucose transport by insulin was significantly reduced both with respect to maximal responsiveness (65% decrease of V_{max}) and sensitivity (2.6-fold increase of ED_{50}) compared with adipocytes cultured in medium containing a low concentration of glucose and no insulin. This reflects insulin resistance of glucose transport. In contrast, both responsiveness and sensitivity of glucose transport toward stimulation by glimepiride were only marginally reduced in insulin-resistant adipocytes (15% decrease of V_{max} ; 1.2-fold increase of ED_{50}) versus control cells. Glimepiride, in combination with glucose and glutamine during the primary culture, caused desensitization of the glucose transport system toward stimulation by insulin, but to a lesser degree than insulin itself (50% reduction of V_{max} ; ninefold increase of ED_{50}). Again, the maximal responsiveness and sensitivity of glucose transport toward stimulation by

glimepiride were only slightly diminished. The presence of glimepiride during primary culture did not antagonize the induction of insulin resistance of glucose transport. The stimulation of glucose transport in insulin-resistant adipocytes by glimepiride is caused by translocation of glucose transporters from low-density microsomes to plasma membranes as demonstrated by subcellular fractionation and immunoblotting with anti-GLUT1 and anti-GLUT4 antibodies. Immunoprecipitation of GLUT4 from ^{32}P - and ^{35}S -methionine-labeled adipocytes revealed that the insulin resistance of GLUT4 translocation is accompanied by increased (three- to fourfold) phosphorylation of GLUT4 in both low-density microsomes and plasma membranes. Short-term treatment of desensitized adipocytes with glimepiride or insulin reduced GLUT4 phosphorylation by ~70 and 25%, respectively, in both fractions. We conclude that glimepiride activates glucose transport by stimulation of GLUT1 and GLUT4 translocation in rat adipocytes via interference at a site downstream of the putative molecular defect in the signaling cascade between the insulin receptor and the glucose transport system induced by high concentrations of glucose and insulin. The molecular site of glimepiride action is related to GLUT4 phosphorylation/dephosphorylation, which may regulate glucose transporter activity and translocation. These in vitro findings implicate an additional mode of sulfonylurea action in the improvement of glucose tolerance of NIDDM patients. *Diabetes* 42:1852–67, 1993

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Received for publication 15 September 1992 and accepted in revised form 8 July 1993.

NIDDM, non-insulin-dependent diabetes mellitus; DMEM, Dulbecco's modified Eagle's medium; HBSS, HEPES-based balanced salt solution; PMSF, phenylmethylsulfonyl fluoride; LDM, low-density microsomes; PM, plasma membrane; GLUT, glucose transporter isoform; STZ, streptozocin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BSA, bovine serum albumin; KRBB, Krebs-Ringer bicarbonate buffer; FCS, fetal calf serum; β -COP, β -coat protein; TCA, trichloroacetic acid; IgG, immunoglobulin G; PKA, protein kinase A; PKC, protein kinase C; IGF-II, insulin-like growth factor II.

The hypoglycemic potency of sulfonylurea drugs in the treatment of NIDDM has been attributed primarily to acute stimulation of the rate of insulin secretion. However, after long-term treatment of NIDDM patients with sulfonylurea compounds, the plasma insulin levels often return to pretreatment levels. This is not accompanied by hyperglycemia in the fasted state and loss of the improved glucose tolerance

(1,2). Euglycemic clamp studies revealed that sulfonylurea compounds increase insulin-induced glucose utilization in normal rats (3) and NIDDM patients (4,5). These observations may be taken as an indication for extrapancreatic effects of sulfonylurea drugs. However, because of the presence of insulin under these experimental conditions it is difficult to determine the reasons for the increased glucose clearance rates. The compounds either directly stimulate rate-limiting steps of glucose metabolism or enhance their insulin sensitivity or improve the overall metabolic situation in peripheral tissues because of increased insulin and reduced glucose levels. In vitro studies are useful to discriminate between these possibilities.

Numerous in vitro studies using normal cells demonstrated that sulfonylurea drugs directly stimulate glucose metabolizing steps, i.e., glycogen synthesis in rat adipocytes (6) and glucose transport in BC3H-1 myocytes (7,8), L6 myocytes (9), 3T3-L1 adipocytes (10), and isolated rat adipocytes (11,12). Furthermore, sulfonylurea drugs enhance the action of exogenous insulin in depancreatized dogs (13) and potentiate the insulin stimulation of muscle glycogen synthase in NIDDM patients (14). The sulfonylurea-induced enhancement of insulin action could be mimicked in vitro using adipocytes and hepatocytes from STZ-induced diabetic rats. Glyburide potentiated insulin-stimulated but not basal glucose transport (15), and tolazamide increased insulin-sensitive but not basal lipogenesis (16). This suggests that in the absence of insulin, sulfonylurea drugs exert no direct effect on insulin-resistant peripheral and liver cells.

However, the insulin-resistant state evolving during the pathogenesis of NIDDM is characterized by hyperinsulinemia. A large body of experimental evidence points to significant differences in the genesis and characteristics of insulin resistance induced and accompanied by hypo- and hyperinsulinemia (17–20). Therefore, the question emerged whether sulfonylurea drugs exert direct effects on peripheral cells that display insulin resistance induced by hyperinsulinemia. Previously, Marshall et al. (21) introduced a model system for the induction of insulin resistance in vitro by incubation of fat cells in a diabetic (hyperinsulinemic and hyperglycemic) milieu characteristic for NIDDM (22). After 20 h of primary culture with high concentrations of glucose, glutamine, and insulin, freshly isolated rat adipocytes exhibited a significantly reduced maximal insulin responsiveness and sensitivity of the initial glucose transport velocity. This state is defined as insulin resistance of glucose transport.

Herein, we used this method to study whether the novel sulfonylurea drug, glimepiride, directly stimulates glucose transport in insulin-resistant rat adipocytes. Glimepiride was chosen because Geisen (23) showed that in dogs this drug had a long-lasting hypoglycemic effect without markedly elevated plasma insulin levels, suggesting a very efficient extrapancreatic action. We observed a significant direct stimulation of glucose transport and GLUT1 and GLUT4 translocation in insulin-resistant adipocytes by this drug. We then studied the possible mechanism of desensitization of the glucose transport system toward insulin and of glucose transport

stimulation by glimepiride. Phosphorylation of components of signal transduction cascades has been implicated as a mechanism of desensitization of diverse signaling pathways (24,25). Therefore, we asked whether the glucose transporter, a component of the insulin signaling cascade, is also a target for phosphorylation during induction of insulin resistance of glucose transport in primary cultured adipocytes. In fact, we observed increased GLUT4 phosphorylation under these conditions. The phosphorylation state of GLUT4 becomes reduced by acute glimepiride treatment. The possible implications for the therapy of NIDDM with sulfonylurea drugs are discussed.

RESEARCH DESIGN AND METHODS

Radiochemicals and affinity-purified [¹²⁵I]protein A (30 mCi/mg), polyclonal and donkey anti-rabbit [¹²⁵I]IgG, nitrocellulose sheets Hybond C extra, and scintillation cocktail ACSII were bought from Amersham-Buchler (Braunschweig, Germany); goat anti-rabbit IgG coupled to alkaline phosphatase were provided by Sigma (Deisenhofen, Germany); dinonylphthalate oil was from Merck (Darmstadt, Germany); reagents for cell culture were obtained from Gibco-BRL (Eggenstein-Leopoldshafen, Germany); collagenase type CLS II was bought from Worthington (Freehold, NJ); protein A-Sepharose was provided by LKB/Pharmacia (Freiburg, Germany); and polyclonal rabbit anti-GLUT4 antibodies raised against purified rat GLUT4 were provided by Calbiochem (Bad Soden, Taunus, Germany). Peptide anti-GLUT4 antibodies were obtained from a rabbit injected with a peptide corresponding to the COOH-terminal 16 amino acids of rat GLUT4 (26) and coupled to keyhole limpet hemocyanin, and the rabbit anti-GLUT1 antiserum was raised against a peptide corresponding to the COOH-terminal 12 amino acids of the human GLUT1 sequence (27). Both antibodies were affinity-purified. Polyclonal anti-rat β -COP and anti-rat Na⁺-K⁺-ATPase (α -subunit) antisera from rabbit were gifts from Dr. T. Kreis, Heidelberg, and Dr. G.V. Jagow, Frankfurt, Germany, respectively. Semisynthetic human insulin and glimepiride (HOE 490) were prepared by the Pharma Synthesis Department of Hoechst Aktiengesellschaft, Frankfurt; glimepiride was dissolved in 25 mM HEPES/KOH (pH 7.4) at a 2 mM stock solution made daily.

Preparation of rat adipocytes. Adipocytes were isolated from epididymal fat pads of 160- to 180-g male Wistar rats under sterile conditions as described previously (28) with the following modifications: Cut tissue pieces were incubated for 20 min at 37°C with collagenase (1 mg/ml) in KRBB, 25 mM HEPES/KOH (pH 7.4), 0.1 mM glucose, 1% wt/vol BSA. The cell suspension was filtered through a 100- μ m nylon screen, washed 3 times by flotation (accumulation in a thin cell layer on top of the medium after centrifugation at 1000 g for 1 min in a swing-out rotor) with DMEM lacking D-glucose and containing 20 mM HEPES, 2% FCS, 1% BSA, 50 U/ml penicillin, 10 mg/ml streptomycin, and then 2 times with HBSS according to Traxinger and Marshall (29). The suspension was adjusted to a final titer of 1×10^5 cells/ml.

Primary culture. Isolated rat adipocytes were maintained in primary culture according to published procedures (29,30) with modifications. Briefly, 10 ml of adipocyte suspension (1×10^5 cells/ml) were incubated at 37°C in sterile 50-ml polystyrene tubes in HBSS containing either 5 mM glucose (glucose medium), 20 mM glucose plus 16 mM glutamine plus 10 nM insulin (glucose/glutamine/insulin medium), 20 mM glucose plus 16 mM glutamine plus 20 μ M glimepiride (glucose/glutamine/glimepiride medium), or various combinations thereof for different periods of time as indicated in the figure legends. Subsequently, the cells were washed 4 times with 50 ml each of HBSS lacking glucose, glutamine, and insulin. After the last wash, the cells were suspended in 2.5 ml of the same medium (final titer 4×10^5 cells/ml) and further incubated for 30 min at 37°C. This procedure causes the almost complete deactivation of the adipocyte glucose transport system, which had been stimulated by insulin during the primary culture, to basal values (as demonstrated by almost identical basal glucose transport activities of cells that have been incubated for 20 h with glucose medium, glucose/glutamine/insulin medium, and glucose/glutamine/glimepiride medium [Fig. 1]). Each of the different adipocyte populations were subjected to the same washing, flotation, and deactivation procedures. Therefore, any reductions of the cell titer and viability were comparable between adipocytes incubated in the different media.

Labeling of adipocytes with ^{32}P i and ^{35}S methionine.

For incubation with ^{32}P i, the isolated adipocytes were suspended in low Pi medium (20 ml per gram of original tissue) composed of 1% (wt/vol) BSA in HBSS containing 0.1 mM sodium phosphate (pH 7.4). Cells (20-ml aliquots, $1-1.5 \times 10^6$ cells/ml) were incubated with ^{32}P (4 mCi per aliquot) for 2 h at 37°C to achieve steady-state labeling with ^{32}P orthophosphate before incubation with insulin or glimepiride. For incubation with ^{35}S methionine, adipocytes (50-ml aliquots, $0.5-0.75 \times 10^6$ cells/ml) were incubated with ^{35}S methionine (0.5 mCi per aliquot) for 2 h at 37°C, subsequently washed twice with HBSS and suspended in HBSS containing 10 mM unlabeled methionine before incubation with insulin or glimepiride. After the end of the incubation, the cells were centrifuged (1000 g, 30 s) and the medium aspirated. The cells were rinsed in HBSS containing protease and phosphatase inhibitors (see below) and finally homogenized at 4°C in 20 mM Tris/HCl (pH 7.4), 1 mM EDTA, 250 mM sucrose containing 100 mM NaF, 100 μ M sodium orthovanadate, 100 mM sodium pyrophosphate, 200 μ M PMSF, 100 μ M benzamide, 20 μ g/ml of leupeptin, pepstatin, aprotinin, and antipain each (buffer A). The cell homogenates were used for subcellular fractionation.

Subcellular fractionation (according to published procedures [31,32] with modifications). Briefly, cells from 12 rats were preincubated in HBSS containing different combinations of glucose, glutamine, insulin, and glimepiride, as indicated in the figure legends, and washed 3 times. After the last washing step, the packed adipocytes (2 ml) were diluted with 50 ml of buffer A and homogenized 10 times in a Teflon-in-glass homogenizer. The cell homogenate was centrifuged (1500 g, 5 min,

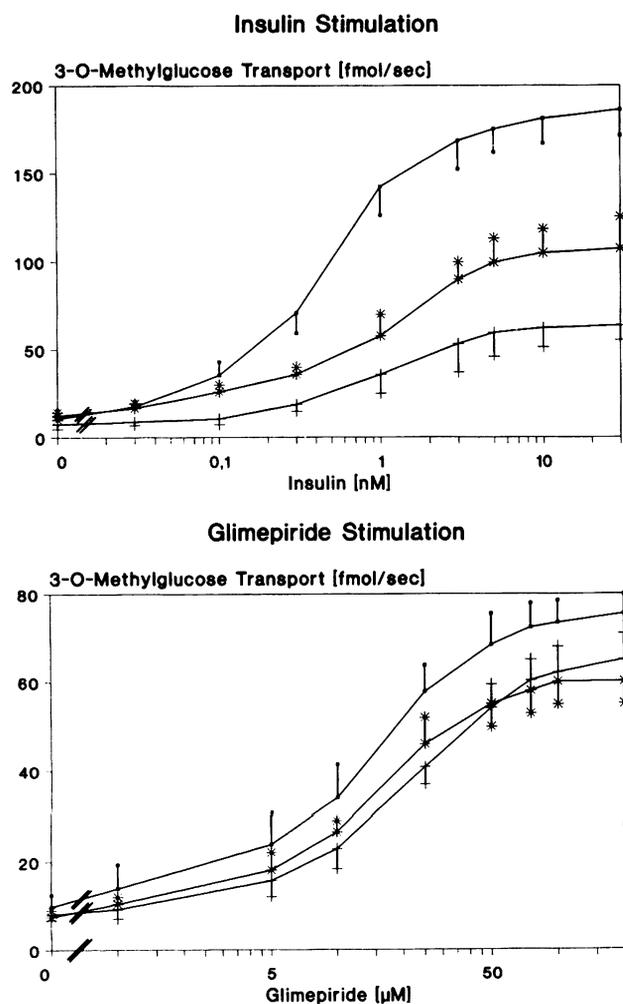


FIG. 1. Stimulation of glucose transport by insulin and glimepiride in normal and insulin-resistant adipocytes. Adipocytes were cultured in glucose medium (■), glucose/glutamine/insulin medium (+), or glucose/glutamine/glimepiride medium (*) for 20 h at 37°C. After full deactivation of the glucose transport system, the cells were incubated with various concentrations of insulin or glimepiride for 20 min at 37°C and assayed for 3-O-methylglucose transport. Each value represents the mean \pm SE of 15 measurements with five different cell preparations. For simplification of the data presentation for this and the following experiments, only one half of the error bar (\pm SE) is shown. The lines indicate the extreme of the SE in either of the two directions calculated for each value.

20°C). The postnuclear supernatant was separated from the fat cake, and the pellet fraction (containing adipocyte ghosts and cell debris) with a needle.

For preparation of the total membrane fraction, the postnuclear supernatant was centrifuged (250,000 g, 90 min, 4°C). The pellet was suspended in 50 ml buffer A, recentrifuged, and finally suspended in buffer A at 0.5 mg protein/ml.

For preparation of LDM, the postnuclear supernatant was sequentially centrifuged (12,000 g, 15 min; 50,000 g, 30 min; 250,000 g, 90 min). The pellet of the last centrifugation was suspended in buffer A, recentrifuged, and finally suspended in buffer A at 1 mg protein/ml.

For preparation of PM, the pellet of the 12,000 g centrifugation was suspended in 35 ml of buffer A and

re-centrifuged (1000 g, 10 min). The supernatant was centrifuged (12,000 g, 20 min). The pellet was washed once, suspended in 5 ml of buffer A, layered onto a 20-ml cushion of 38% (wt/vol) sucrose, 20 mM Tris/HCl (pH 7.4), 1 mM EDTA, and centrifuged (110,000 g, 60 min, 4°C). The membranes at the interface between the two layers (1 ml) were removed by suction, diluted with three volumes of buffer A, and layered on top of an 8-ml cushion of 28% Percoll, 250 mM sucrose, 20 mM Tris/HCl (pH 7), 1 mM EDTA. After centrifugation (45,000 g, 30 min, 4°C), the PMs were withdrawn from the lower third of the gradient (1 ml) with a Pasteur pipet, diluted with 10 volumes of buffer A, and re-centrifuged (250,000 g, 90 min, 4°C). The pellet was suspended, re-centrifuged, and finally dissolved in buffer A at 0.5 mg protein/ml. All samples were stored at -80°C.

Control experiments were performed using 5'-nucleotidase and lipoprotein lipase activity as PM markers and rotenone-insensitive cytochrome *c* reductase and glucose-6-phosphatase as microsomal markers (microsomes derived from endoplasmic reticulum and Golgi stacks cofractionated with LDM). They demonstrated that the observed alterations in GLUT distribution between PMs and LDMs after primary culture with glucose medium, glucose/glutamine/insulin medium, glucose/glutamine/glimepiride medium, or short-term treatment with insulin or glimepiride are not attributable to different recoveries of PM and microsomal membrane protein or variations in cross contamination of the membrane fractions isolated from the differently treated cells (data not shown).

Assay for glucose transport. Basal, insulin-, and glimepiride-stimulated transport of glucose was measured after incubation of 200 μ l of adipocytes in HBSS (4×10^5 cells/ml) with different concentrations of insulin and glimepiride, respectively, (complete dose-response curves) in 15-ml polystyrene tubes for 20 min at 37°C. Initial rates of glucose transport were determined by addition of 50 μ l HBSS containing either 100 μ M 2-deoxy-D-[1- 14 C]glucose (0.5 μ Ci) or 100 μ M 3-O-methyl-D-[1- 14 C]glucose (0.2 μ Ci). After 5-min (deoxyglucose) or 30-s (methylglucose) incubation at room temperature, the reaction was stopped by addition of cytochalasin B (final concentration 50 μ M) and immediate separation of the cells (200- μ l aliquots) from the medium by centrifugation through 150 μ l of dinonylphthalate oil in plastic tubes. The tubes were then cut through the oil layer and the adipocytes counted for radioactivity in 10 ml of scintillation cocktail ACSII. Glucose uptake by diffusion and trapping of glucose in the intercellular spaces of the packed adipocytes were corrected by performing control incubations in which cytochalasin B was present before addition of the labeled glucose. These radioactivity values were subtracted from the total cell-associated radioactivity.

It has been demonstrated previously (33,34) that the initial rates of glucose transport can be accurately determined with both hexose derivatives, thus yielding comparable stimulatory factors of deoxyglucose and methylglucose transport by insulin. Nevertheless, the key findings (alterations of the dose-response curves for

insulin and glimepiride after primary culture of adipocytes) obtained with 2-deoxyglucose were confirmed with 3-O-methylglucose (Fig. 1). The use of this glucose derivative excludes any influence of the subsequent hexokinase reaction on the transport kinetics. Transport of 3-O-methylglucose was assayed over 30 s and thus led to underestimation of the V_{max} and ED_{50} values for insulin-stimulated transport. Nevertheless, the differences in the initial transport velocities between 2-deoxyglucose and 3-O-methylglucose are rather small, and the stimulatory factors for insulin are close to published values (34). Moreover, underestimations for glucose transport stimulation by glimepiride would not affect the main conclusions of this study.

Calculations of sensitivity and responsiveness toward insulin and glimepiride were performed by running complete dose-response curves for insulin (0.03–30 nM) and glimepiride (1–200 μ M). Maximal insulin and glimepiride stimulation of glucose transport (V_{max}) was determined at 10 nM and 50 μ M, respectively. A decrease of V_{max} with no change in the sensitivity toward the corresponding compound is defined as decreased responsiveness. An increase in the concentrations of insulin and glimepiride necessary for ED_{50} of glucose transport with no change in the maximal responsiveness is defined as decreased sensitivity of the glucose transport system toward insulin and glimepiride. In this study, the terms insulin-resistant and desensitized adipocytes are used for cells that after primary culture display reduced insulin responsiveness or sensitivity or both.

Western blotting of GLUT1 and GLUT4. Proteins were precipitated with 5% TCA (30 min on ice, 10,000 g, 5 min; followed by three acetone washes and evaporation), dissolved in Laemmli buffer containing 2% mercaptoethanol, heated (95°C, 5 min), separated by SDS-PAGE, and transferred electrophoretically to nitrocellulose sheets (35). The blots were saturated for 4 h at room temperature with PBS containing 5% defatted milk powder. After addition of anti-GLUT1 (1:200) or anti-GLUT4 (1:1000) antiserum, the incubation was continued for 16 h at 4°C. Subsequently, the sheets were washed 4 times (30 min each) in PBS containing 1% TX-100 and then incubated with [125 I]protein A (0.5 μ Ci/ml) in PBS containing 5% milk powder. After four washes (see above), the blots were dried and subjected to autoradiography. Immunoreactivity was quantitated by cutting rectangular strips, corresponding to the locations of either GLUT1 or GLUT4 on the developed films, from the nitrocellulose filters and counting the filter strips in a γ -counter. Specific binding was determined after subtracting half of the sum of background radioactivities present on size-matched strips of the filter cut from an area above and below each immunoreactive transporter band.

Immunoprecipitation of radiolabeled GLUT4. PMs, LDMs, and cell homogenates from 32 Pi- or [35 S]methionine-labeled adipocytes were solubilized in Laemmli buffer (5 min, 95°C) at 0.5 mg protein per ml. Equivalent sample volumes (up to 50 μ l) were diluted with 1 ml of buffer A containing 1% TX-100, 150 mM NaCl and protease and phosphatase inhibitors, and centrifuged (10,000 g, 5 min). The supernatant was incubated with 50

μ l of anti-rat GLUT4 peptide antibodies that had been adsorbed to protein A-Sepharose (10 μ l of a 1:500 antiserum dilution was incubated with 50 mg protein A-Sepharose beads in 0.5 ml of the buffer described above for 2 h at 4°C under constant shaking. The beads were pelleted by centrifugation, washed twice with buffer, and resuspended in 1 ml of buffer). After 1 h at 4°C under constant shaking, the beads were collected by centrifugation (10,000 g, 30 s), washed sequentially with 1 ml each of buffer A containing 1% TX-100/100 mM NaCl and 0.1% TX-100/50 mM NaCl and 0.1% TX-100. The final pellet with the immunocomplex-containing beads was solubilized in 50 μ l of sample buffer (5 min, 95°C) and centrifuged. The supernatant was analyzed by SDS-PAGE and fluorography.

Miscellaneous procedures. Western blotting with rabbit anti- β -COP, anti- Na^+ - K^+ -ATPase, and anti-GLUT4 (total protein) antibodies was performed as described above for anti-GLUT4 peptide antibodies but using 1:500, 1:150, and 1:250 antiserum dilutions, respectively. This was followed by decoration of the washed nitrocellulose filters with anti-rabbit [125 I]IgG (0.2 μ Ci/ml) and autoradiography for β -COP (36) and Na^+ - K^+ -ATPase (37), and with anti-rabbit IgG coupled to alkaline phosphatase and chemiluminescence assay for GLUT4. SDS-PAGE using 5% stacking and 15% running gels (38,39), autoradiography using Kodak X-OMAT AR films, protein determination with the amidoblack staining method (40), and assays for 5'-nucleotidase, lipoprotein lipase (41), rotenone-insensitive cytochrome c reductase, and glucose-6-phosphatase (31,42) were performed according to published procedures.

RESULTS

Primary culture of rat adipocytes can induce insulin resistance of glucose transport. As Marshall et al. (21,29) demonstrated previously and as is confirmed in this study, primary culture of isolated rat adipocytes in the presence of high concentrations of glucose and insulin leads to a marked desensitization of the glucose transport system. In cells incubated for 20 h in the presence of 20 mM glucose, 16 mM glutamine, and 10 nM insulin (hereafter called insulin-resistant or desensitized cells), both the insulin sensitivity (Fig. 1A: ED_{50} for insulin 1.85 nM in insulin-resistant cells vs. 0.45 nM in normal cells) and maximal insulin responsiveness (Fig. 1A: 61 fmol/s in insulin-resistant cells vs. 186 fmol/s in normal cells) of 3-O-methylglucose transport are decreased compared with cells maintained at 5 mM glucose during the same period of time (hereafter called normal cells).

Glimepiride stimulates glucose transport in normal and desensitized adipocytes. The sulfonylurea drug, glimepiride, stimulates initial 3-O-methylglucose transport velocity in normal rat adipocytes (Fig. 1B) in a concentration-dependent manner (ED_{50} = 16.5 μ M) up to eightfold (75 fmol/s). This comprises ~50% of the maximal insulin stimulation. With insulin-resistant cells, the dose-response curve for glimepiride stimulation of glucose transport is only slightly shifted to the right

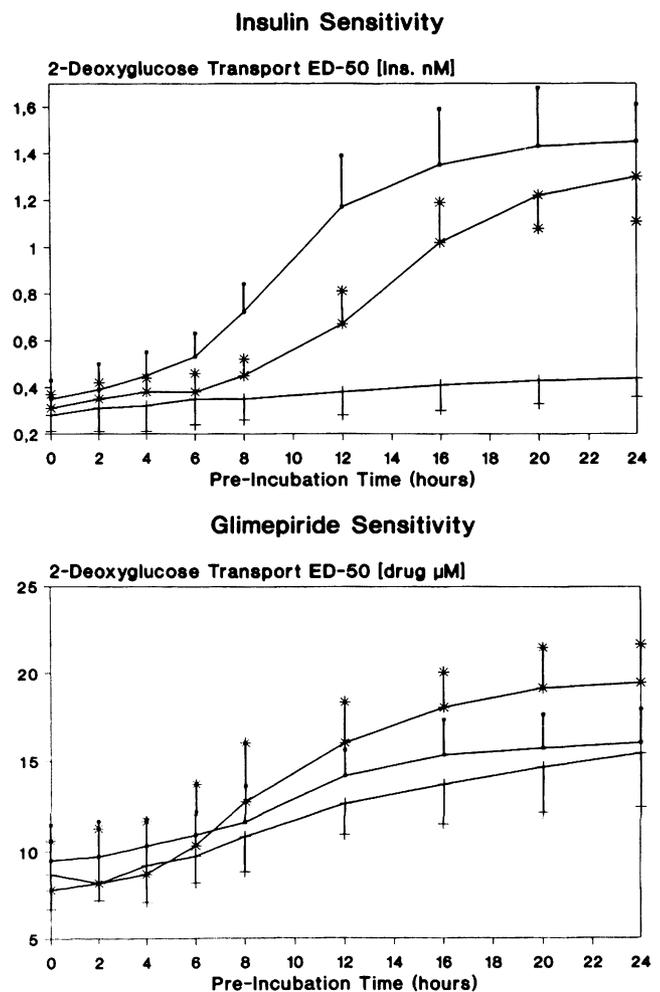


FIG. 2. Time course of the loss of insulin and glimepiride sensitivity of glucose transport. Adipocytes were cultured in glucose medium (+), glucose/glutamine/insulin medium (■), or glucose/glutamine/glimepiride medium (*) for various periods of time at 37°C. After full deactivation of the glucose transport system, the cells were incubated with a full range of insulin (1–30 nM) or glimepiride concentrations (1–200 μ M) for 20 min at 37°C and assayed for 2-deoxyglucose transport. Insulin and glimepiride sensitivity (ED_{50}) of the glucose transport system was calculated at the indicated times from the insulin and glimepiride dose-response curves for each of the three adipocyte populations. Each value represents the mean \pm SE of 16 measurements with four different cell preparations.

(ED_{50} = 22.5 vs. 16.5 μ M) with a concomitant 15% decrease of the maximal transport velocity.

The extent of resistance toward insulin or glimepiride is dependent on the duration of the primary culture and insulin concentration (Figs. 2–4). The development of reduced insulin responsiveness of 2-deoxyglucose transport (Fig. 3A; onset after 2 h) precedes the appearance of decreased insulin sensitivity (Fig. 2A; onset after 6 h). After 20 h incubation, both parameters are altered most pronouncedly. Similar data were obtained with 3-O-methylglucose (compare with Fig. 1A). Primary culture with 5–100 nM causes a significant reduction of the insulin responsiveness of 3-O-methylglucose transport (Fig. 4A; ED_{50} = 4.5 nM for half-maximal loss of responsiveness). The maximal effect requires 50 nM insulin. Therefore, 20 h primary culture in the presence of 50 nM

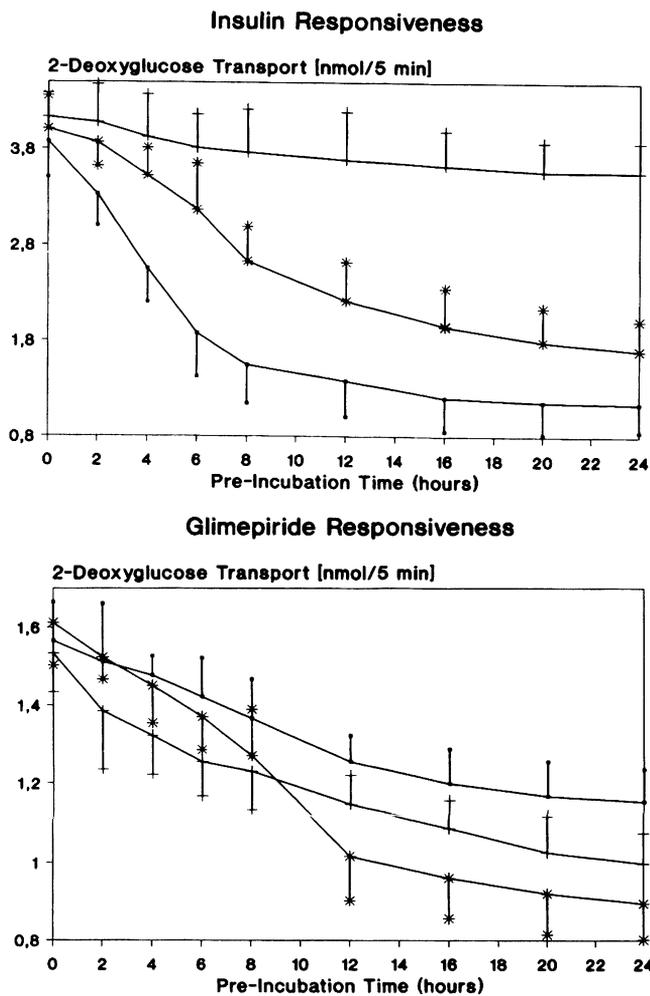


FIG. 3. Time course of the loss of insulin and glimepiride responsiveness of glucose transport. Adipocytes were cultured in glucose medium (+), glucose/glutamine/insulin medium (■), or glucose/glutamine/glimepiride medium (*) for various periods of time. After full deactivation of the glucose transport system, the cells were incubated with 10 nM insulin or 20 μ M glimepiride for 20 min at 37°C and subsequently assayed for 2-deoxyglucose transport. Each value represents the mean \pm SE of 15 measurements with five different cell preparations.

insulin were chosen for all subsequent experiments. In contrast, there is no significant loss of glimepiride responsiveness (Figs. 3B and 4A) and sensitivity (Fig. 2B) during primary culture (Figs. 2B and 3B) with any insulin concentration (Fig. 4A) if one takes into account the general loss of glucose transport stimulation by insulin or glimepiride during the incubation period.

Glimepiride can substitute for insulin in desensitizing glucose transport in primary cultured adipocytes. It has been suggested that the requirement of insulin for desensitization of isolated rat adipocytes by high concentrations of glucose, glutamine, and insulin is based solely on the increased influx of glucose (21). Because glimepiride stimulates glucose transport to about half-maximal insulin levels, we asked whether this sulfonylurea drug also causes insulin resistance of glucose transport when present during primary culture. Figure 1A shows that glimepiride causes a marked reduction of the

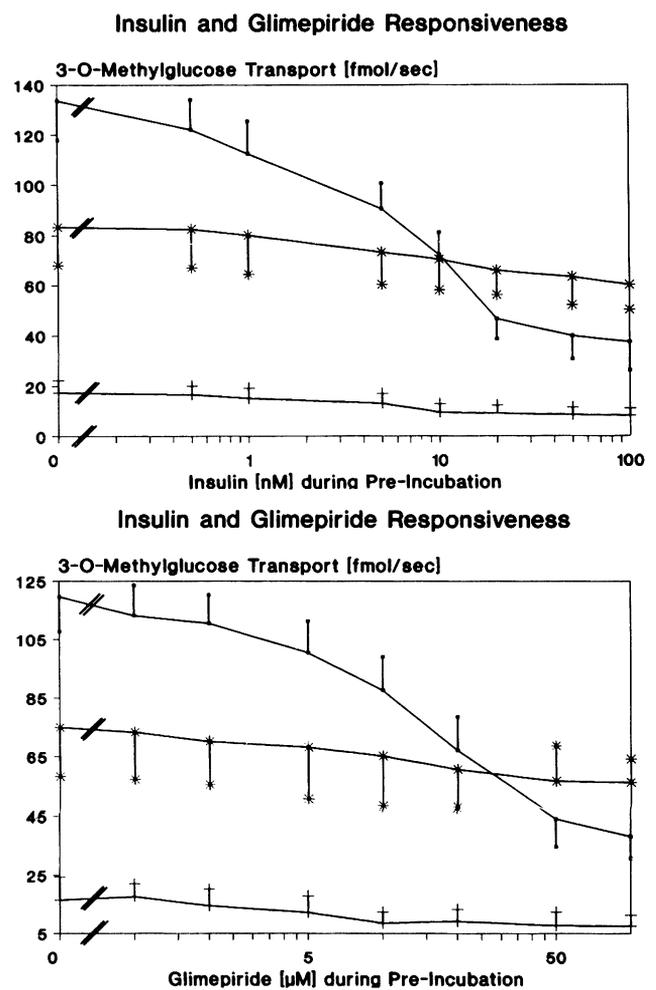


FIG. 4. Dependency of the insulin and glimepiride responsiveness of glucose transport on the insulin and glimepiride concentration during long-term incubation. Adipocytes were cultured in medium containing 20 mM glucose plus 16 mM glutamine plus various concentrations of insulin or glimepiride for 20 h at 37°C. After full deactivation of the glucose transport system, one portion of the cells was incubated directly in the absence (+) or presence of 1.85 nM insulin (■) or 20 μ M glimepiride (*) for 20 min at 37°C and assayed for 3-O-methylglucose transport. Each value represents the mean \pm SE of six measurements with two different cell preparations.

maximal insulin-stimulated 3-O-methylglucose transport velocity (102 fmol/s in insulin-resistant cells vs. 192 fmol/s in normal cells) and an increase of the ED₅₀ for insulin (1.1 nM in insulin-resistant cells vs. 0.45 nM in normal cells). The most pronounced reductions of insulin responsiveness and sensitivity become evident after incubation for 20 h (Figs. 2A and 3A). The time course for the glimepiride-induced insulin resistance lags ~2–4 h behind the insulin effect in both its onset and time to reach maximum. Interestingly, as insulin present during primary culture causes desensitization of the glucose transport toward insulin but not glimepiride, long-term treatment with this drug provokes insulin resistance of 3-O-methylglucose transport but only marginally impairs transport stimulation by the drug itself (Fig. 1B). The detailed analysis revealed that glimepiride causes an ~50% reduction of the maximal insulin-stimulated 2-deoxyglu-

TABLE 1
Effect of glimepiride on the induction of insulin resistance of glucose transport

| 3-O-methylglucose transport | | | | |
|--------------------------------|-------------|----------------------------------|-------------|-------------------------------|
| Percentage of maximal response | | Percentage increase of ED_{50} | | Conditions of primary culture |
| Insulin | Glimepiride | Insulin | Glimepiride | |
| 55.7 ± 6.5 | 89.4 ± 9.9 | 252 ± 20.3 | 110 ± 13.4 | Glc/Gln/Gli |
| 32.9 ± 5.5 | 83.1 ± 7.8 | 291 ± 26.7 | 141 ± 10.2 | Glc/Gln/Ins |
| 31.2 ± 4.1 | 74.3 ± 6.1 | 314 ± 35.7 | 149 ± 16.1 | Glc/Gln/Ins/Gli |

Data are means ± SE of six measurements with different cell preparations. Adipocytes were cultured in glucose/glutamine/glimepiride medium or glucose/glutamine/insulin medium in the absence or presence of 50 μ M glimepiride (glucose/glutamine/insulin/glimepiride) for 20 h at 37°C. After full deactivation of the glucose transport system, portions of the cells were incubated with a full range of concentrations of either insulin (0.02–30 nM) or glimepiride (1–200 μ M) for 20 min at 37°C and then assayed for 3-O-methylglucose transport. The maximal initial transport velocities (30 nM insulin or 50 μ M glimepiride, respectively) and the increments of the ED_{50} values for insulin and glimepiride were calculated for each population of adipocytes as the percentage of the corresponding values for cells kept in glucose medium (set at 100%). Glc, glucose; Gln, glutamine; Ins, insulin; Gli, glimepiride.

cose transport (Fig. 3A) and a 2.9-fold increase of the ED_{50} for insulin (Fig. 2A). The maximal glimepiride-stimulated glucose transport (Fig. 3B) and the ED_{50} values for glimepiride (Fig. 2B) showed only a very slight decrease and increase, respectively, after various periods of primary culture with glimepiride. Figure 4B shows the dependency of the glimepiride-induced insulin resistance on the drug concentration. The concentration required for half-maximal inhibition is 13.5 μ M and thus closely correlated to the ED_{50} value for stimulation of glucose transport by glimepiride.

In accordance with the impairment of the insulin-stimulated glucose transport by long-term insulin and glimepiride treatment, the drug does not antagonize the desensitization of the glucose transport system toward insulin when present during the primary culture together with insulin (Table 1). The maximal glucose transport velocity, responsiveness, and ED_{50} values for insulin do not differ significantly between insulin-resistant adipocytes cultured in the absence or presence of glimepiride.

Glimepiride stimulates translocation of GLUT1 and GLUT4. Next we were interested in the mechanism of glucose transport stimulation by glimepiride in normal and insulin-resistant adipocytes and of insulin resistance induced by long-term glimepiride treatment. Drug stimulation of glucose transport may be attributable to recruitment of GLUT molecules from LDMs to PMs as has been well established for insulin (43,44) and to increased synthesis of GLUT. Therefore, we studied whether glimepiride mimicks the insulin-stimulated increase of cell surface expression of GLUT1 and GLUT4 (45). For studying the translocation of GLUT1 and GLUT4, cultured rat adipocytes were treated with insulin or glimepiride and then fractionated into LDMs and PMs. Their relative purity is proved by the enrichment and little mutual cross-contamination of typical marker proteins for PMs, i.e., lipoprotein lipase, 5'-nucleotidase, Na^+K^+ -ATPase (α -subunit), and for microsomes (which cofractionate but are not identical with LDM), i.e., glucose-6-phosphatase, rotenone-insensitive cytochrome *c* reductase, and β -COP, as revealed by enzymic assays (data not shown) and Western blot analysis (Table 2). The latter demonstrates ~10/5-fold enrichment of Na^+K^+ -ATPase/

β -COP in the PM and LDM fractions, respectively, and vice versa. PM and LDM contain only 2–4% of the total β -COP and Na^+K^+ -ATPase protein, respectively. The number of GLUT molecules in both membrane fractions was assessed by Western blotting with specific anti-GLUT1 and anti-GLUT4 peptide antisera. For evaluation of possible effects on gene expression of GLUT1 and GLUT4, total cellular membrane fractions, from which PMs and LDMs were ultimately derived, were prepared and tested for anti-GLUT1 and anti-GLUT4 immunoreactivity.

Incubation of isolated rat adipocytes with insulin increases the amount of GLUT4 and GLUT1 in isolated PM by ~5- to 6-fold and 2-fold, respectively (Fig. 5A and B, lanes 3 and 6; Fig. 6A and B). In contrast, the amount of both GLUT isoforms decreases in the LDM fraction (lanes 2 and 5). This reflects the insulin-induced redistribution of GLUT4 and GLUT1 from internal stores to the cell surface. Translocation of GLUT is provoked also by glime-

TABLE 2
Distribution of marker proteins in adipocyte membranes

| Fraction | Total protein (mg) | Relative immunoreactivity (arbitrary units) | | Relative yield (%) | |
|-----------------|--------------------|---|--------------|--------------------|--------------|
| | | Na^+K^+ -ATPase | β -COP | Na^+K^+ -ATPase | β -COP |
| Total membranes | 8.04 | 1 | 1 | 100 | 100 |
| PM fraction | 0.39 | 9.65 | 0.76 | 46.8 | 3.6 |
| LDM fraction | 1.02 | 0.25 | 5.42 | 2.5 | 55.3 |

Rat adipocytes were fractionated into total membranes, LDM, and PM. One hundred micrograms of protein of each fraction was separated on SDS-PAGE, transferred to nitrocellulose, and immunodecorated with rabbit anti- Na^+K^+ -ATPase and anti- β -COP antibodies. After incubation with [¹²⁵I]anti-rabbit IgG, antibodies and autoradiography nitrocellulose strips corresponding to the visualized Na^+K^+ -ATPase and β -COP protein bands were counted for radioactivity in a γ -counter. Correction for background binding was done as described for Fig. 6. The radioactivity measured for 100 μ g total membrane protein was set at 1 arbitrary unit (relative immunoreactivity), and the radioactivity calculated for the total adipocyte membranes was set at 100% (relative yield).

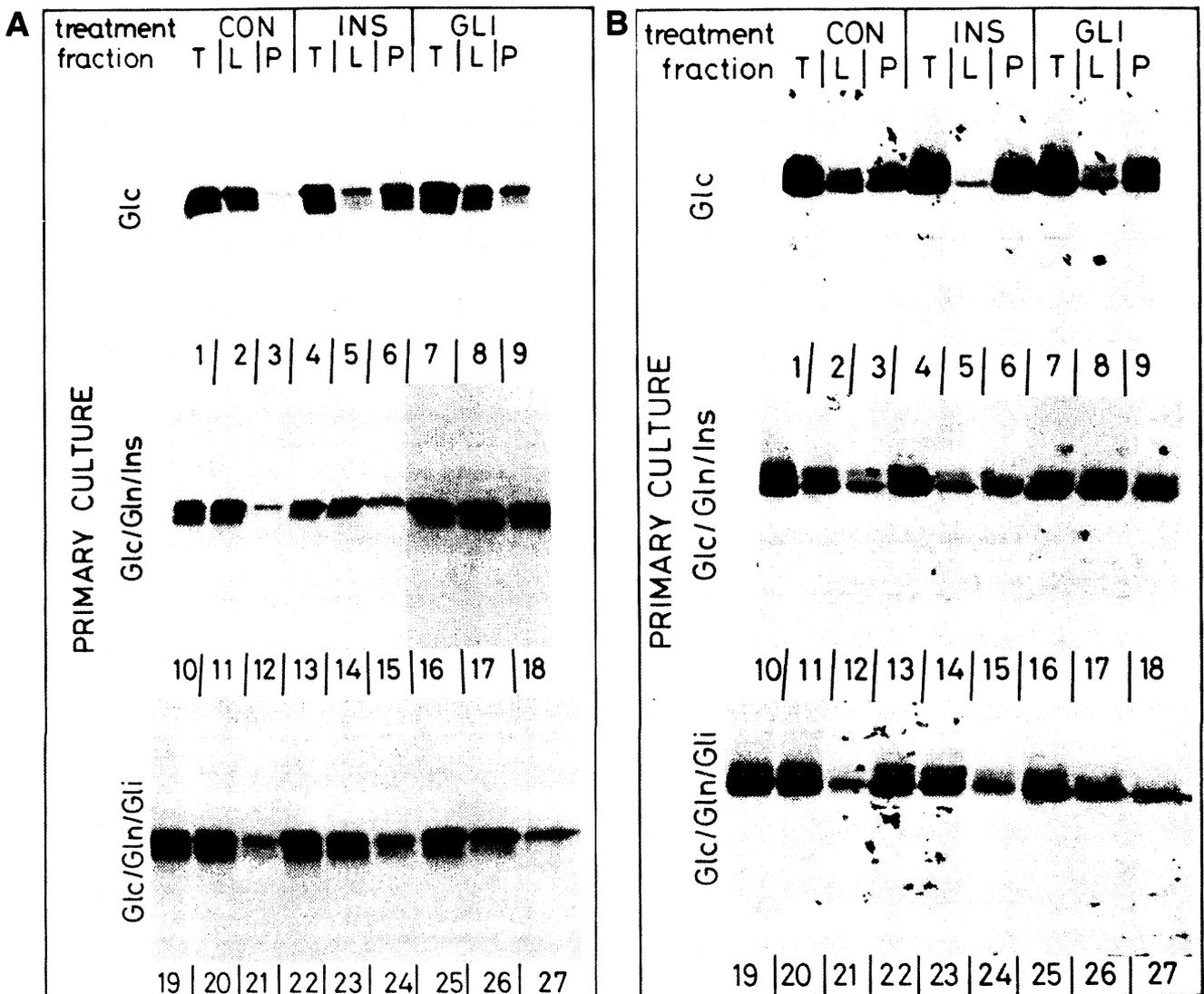


FIG. 5. Effect of primary culture and treatment of adipocytes with insulin and glimepiride on GLUT translocation. Adipocytes were cultured in glucose medium, glucose/glutamine/insulin medium, or glucose/glutamine/glimepiride medium for 20 h at 37°C. After full deactivation of the glucose transport system, the cells were incubated in the absence (CON) or presence of 10 nM insulin (INS) or 20 μ M glimepiride (GLI) for 20 min at 37°C. Subsequently, the cells were homogenized. From one half of the homogenate total membranes were prepared (T). The other half was fractionated into LDM (L) and PM (P) as described in METHODS. Equivalent amounts of protein (T, 200 μ g; L, 18.4 μ g; P, 20.6 μ g; the relative subcellular distribution of the protein content did not differ significantly between the three adipocyte populations and after stimulation with insulin or glimepiride; data not shown) were separated on SDS-PAGE, and the amount of GLUT1 and GLUT4 in each fraction was determined separately by immunoblotting with anti-GLUT1 and anti-GLUT4 antibodies, respectively. Autoradiograms of the immunoblots with anti-GLUT4 (A) and anti-GLUT1 antibody (B) subsequently decorated with [¹²⁵I]protein A for a typical experiment repeated three times are shown.

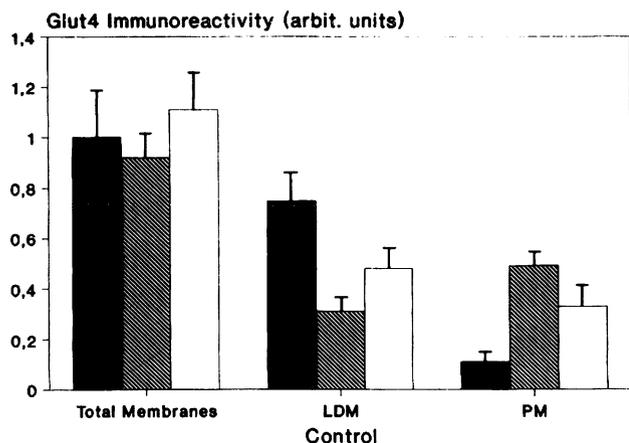
piride (25 μ M) with ~50% efficiency compared with insulin (Fig. 5A and B, lanes 8 and 9; Fig. 6A and B). This explains, at least in part, stimulation of glucose transport by the drug.

Glimepiride-stimulated GLUT translocation is only slightly reduced in desensitized adipocytes. Desensitization of the glucose transport system leads to a markedly impaired insulin-stimulated increase of the GLUT4 and GLUT1 number in the PM and decrease in the LDM fraction (Fig. 5A and B, lanes 14, 15, 23, and 24; Fig. 6C and D). The stimulation factors for GLUT4 and GLUT1 translocation in insulin-resistant versus normal adipocytes, calculated as the ratio of the amount of immunoreactive material (arbitrary units in Fig. 6) in the PM

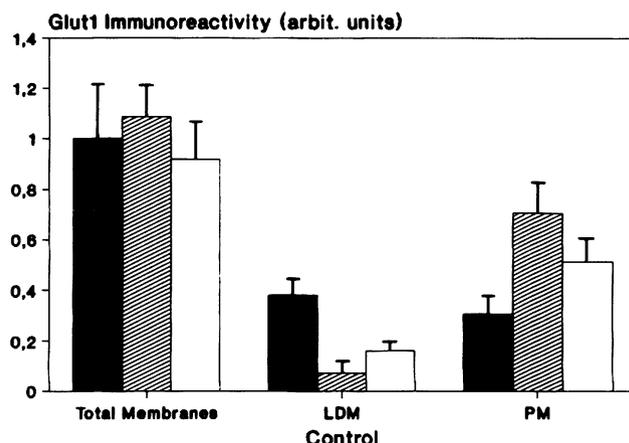
fraction in the stimulated and basal state, were diminished (2.7 and 2.8 vs. 5.4). In contrast, the stimulation factors of glimepiride are only slightly diminished in insulin-resistant versus normal adipocytes, irrespective of whether the resistance has been induced by insulin (2.6 vs. 3.3) or glimepiride (3.1 vs. 3.3; Fig. 5A and B, lanes 17, 18, 26, and 27; Fig. 6E and F). This correlates well with the decreased insulin but almost unaltered glimepiride responsiveness and sensitivity of the glucose transport in desensitized adipocytes.

The total number of GLUT4 and GLUT1 determined in a total membrane fraction does not change during short-term incubation with either insulin or glimepiride (Fig. 5, lanes 1, 4, and 7; Fig. 6A and B). In contrast, the amount

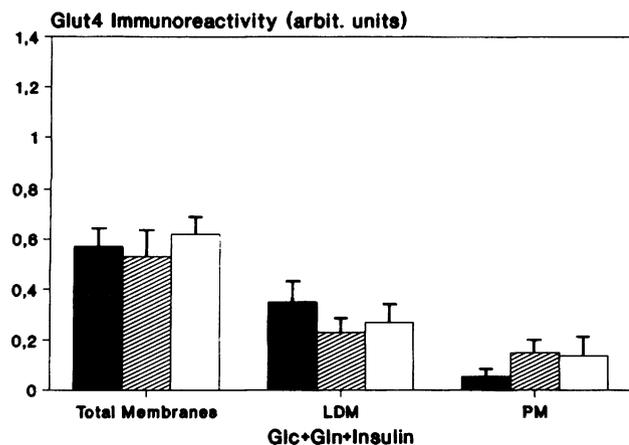
A Responsiveness of Glut4 Translocation



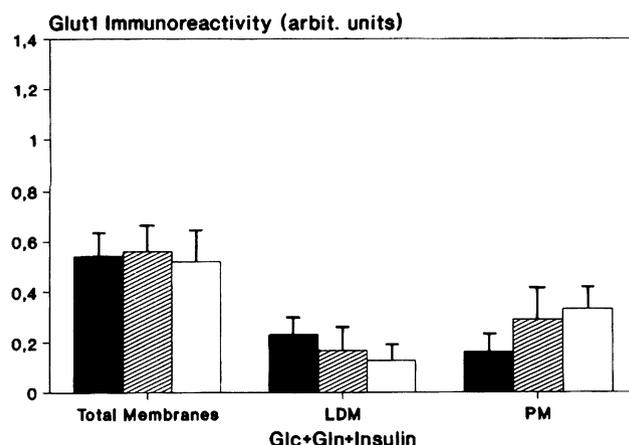
B Responsiveness of Glut1 Translocation



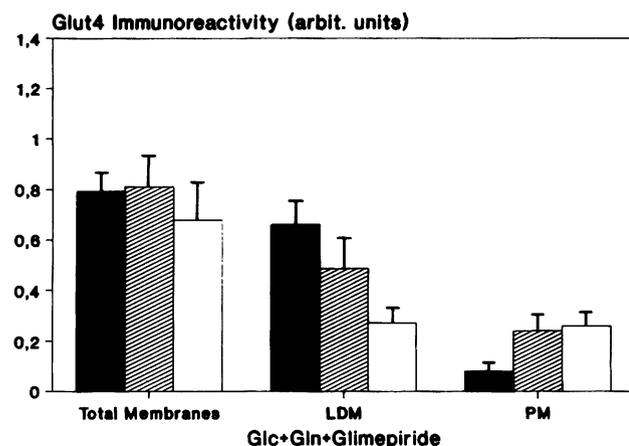
C Responsiveness of Glut4 Translocation



D Responsiveness of Glut1 Translocation



E Responsiveness of Glut4 Translocation



F Responsiveness of Glut1 Translocation

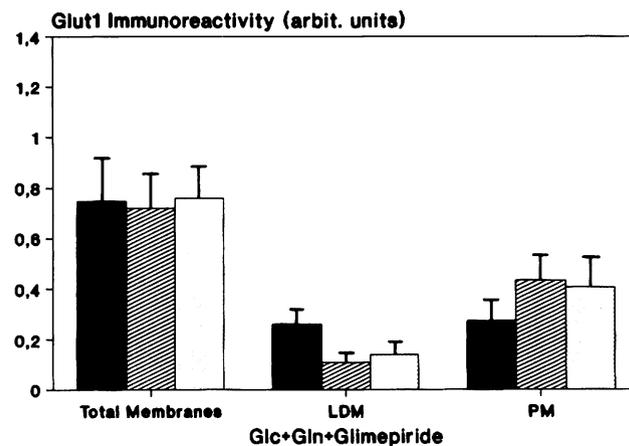


FIG. 6. Quantitation of the immunoblot data of Fig. 5. The radioactive filter strips were sliced and counted for radioactivity in a γ -counter. The amount of GLUT4 and GLUT1, respectively, in total membranes of adipocytes cultured in glucose medium and in the basal state (without insulin and glimepiride stimulation) was set at 1.0 arbitrary units. Each value represents the mean of four fractionations with different cell preparations. A, C, E: Anti-GLUT4; B, D, F: anti-GLUT1; A, B: glucose medium; C, D: glucose/glutamine/insulin medium; E, F: glucose/glutamine/glimepiride medium. (■), Basal; (▨), insulin stimulation; (□), glimepiride stimulation.

of GLUT4 and GLUT1 is significantly reduced after primary culture of the adipocytes with glucose and glutamine in the presence of either insulin (Fig. 6C and D) or, to a lesser degree, glimepiride (Fig. 6E and F). Subsequent acute challenge of insulin or glimepiride of insulin-resistant cells does not affect total GLUT4 and GLUT1 protein (Fig. 6C-F).

Glucosamine is involved in desensitization of glucose transport by insulin and glimepiride. Marshall et al. (21) have demonstrated that glucosamine is able to substitute for glucose and glutamine in inducing desensitization of the glucose transport system. Therefore, we studied whether glimepiride is able to circumvent the glucosamine-induced insulin resistance. Figure 7A confirms that insulin in combination with glucose plus glutamine, but not with either compound alone, reduces the maximal insulin responsiveness but leaves the responsiveness toward glimepiride largely unimpaired. Glucosamine, but only in combination with insulin, reduces the maximal insulin but not glimepiride responsiveness. These observations indicate that glimepiride bypasses the desensitization of the glucose transport system at a site downstream of the formation of glucosamine from glucose and glutamine.

Next we studied whether glimepiride, like insulin, desensitizes glucose transport via formation of glucosamine. Glimepiride in combination with glucosamine present during primary culture causes an ~50% reduction of the maximal insulin responsiveness similar to the effect of glucose plus glutamine (Fig. 7B). Glimepiride combined with either glucose or glutamine alone leads to only a slight impairment. Under all conditions of primary culture, the maximal loss of glimepiride responsiveness does not exceed 20%. The ability of glucosamine to induce insulin resistance if present together with insulin or glimepiride strongly suggests that both agents use similar mechanisms for desensitization of glucose transport that may involve increased formation of hexosamine derivatives.

Desensitization of glucose transport in primary cultured adipocytes is accompanied by increased phosphorylation of GLUT4. Finally, we studied whether increased phosphorylation of GLUT molecules represents the molecular mechanism for their desensitization toward insulin as may be deduced from some recent observations (46,47,47a). Therefore, we investigated the phosphorylation state of GLUT4 in rat adipocytes during desensitization of the glucose transport system. For this, adipocytes were cultured for 20 h with high levels of glucose, glutamine, and insulin. Subsequently, the cells were incubated in the presence of either ^{32}P i or ^{35}S methionine under the same conditions for 2 h to achieve steady-state labeling of the cells before short-term treatment with insulin or glimepiride. After immunoprecipitation of solubilized total membranes, LDM and PM fractions with anti-GLUT4 antibodies, the ^{35}S methionine- and ^{32}P i-labeled GLUT4 molecules were analyzed by SDS-PAGE. As can be seen from the fluorograms of the gels (Fig. 8), induction of insulin resistance by primary culture of the adipocytes in glucose/glutamine/insulin medium reduces the total amount of GLUT4

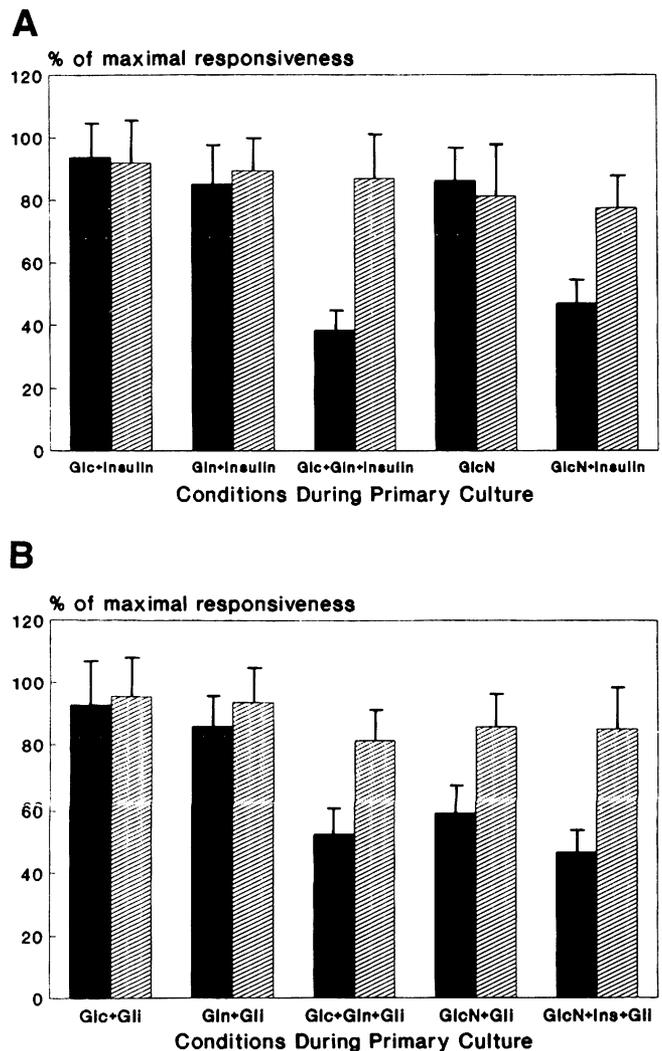


FIG. 7. Effect of glucosamine during primary culture on insulin (A) and glimepiride (B) responsiveness of glucose transport. Adipocytes were cultured in medium containing various combinations of insulin (10 nM), glimepiride (50 μM), glucose (20 mM), glutamine (16 mM), and glucosamine (2 mM) for 20 h at 37°C as indicated. After full deactivation of the glucose transport system, portions of the cells were incubated in the presence of 10 nM insulin (■) or 50 μM glimepiride (▨) for 20 min at 37°C and subsequently assayed for 2-deoxyglucose transport. The initial transport velocity of cells cultured in glucose medium during the 20-h period was set at 100% of maximal responsiveness for insulin (■) and glimepiride (▨) stimulation, respectively. Each value represents the mean \pm SE of nine measurements with three different cell preparations.

protein synthesized during 2 h (compare Fig. 8A and B, lanes 1, 4, and 7) and impairs the insulin-stimulated redistribution of GLUT4 from LDM to PM (compare Fig. 8A and B, lanes 5 and 6). The quantitative evaluation of the data (Fig. 8C and 8D) proves that the subcellular distribution of ^{35}S methionine-labeled and immunoprecipitated GLUT4 closely reflects the amount of GLUT4 in the LDM and PM fractions as revealed by immunoblotting (Fig. 5). Despite the loss of GLUT4 protein in desensitized cells, the total amount of ^{32}P i-labeled GLUT4 increases 2.9-fold in these cells (Fig. 8A and B, lanes 10, 13, and 16; 8E and F). Correction for the different amounts of immunoprecipitated ^{35}S methionine-labeled

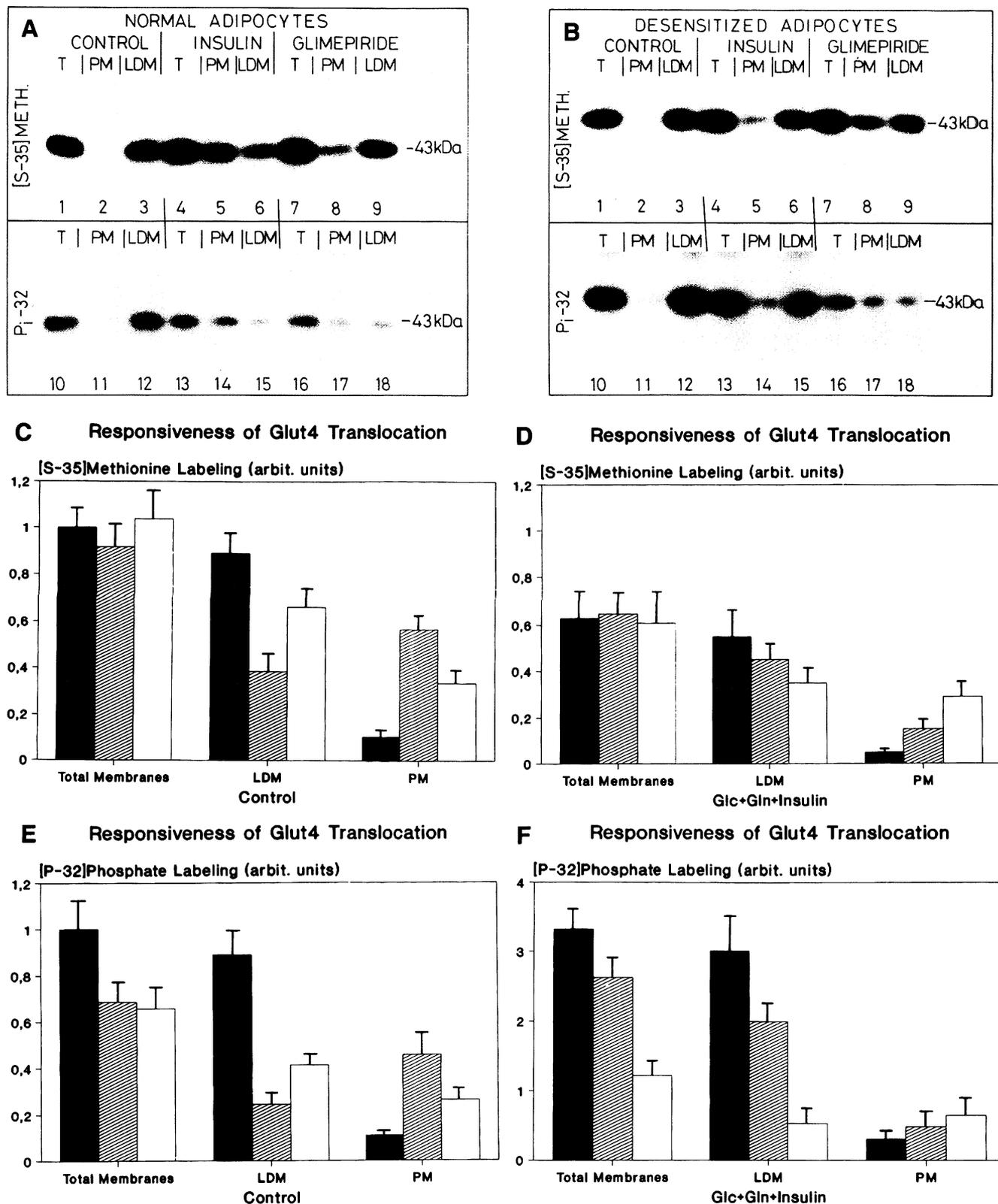


FIG. 8. Effect of primary culture and treatment with insulin and glimepiride of adipocytes on the phosphorylation of GLUT4. Adipocytes were cultured in glucose medium (normal, *A, C, E*) and glucose/glutamine/insulin medium (desensitized, *B, D, F*) containing 0.1 mM sodium phosphate for 20 h at 37°C. Subsequently, the incubation was continued with [³⁵S]methionine (*lanes 1–9*) or ³²P (*lanes 10–18*) for 2 h. After full deactivation of the glucose transport system, the washed cells were incubated in the absence (*Control*) or presence of 10 nM insulin and 20 μM glimepiride for 20 min at 37°C (during the periods of glucose transport deactivation and subsequent treatment with insulin/glimepiride, the same specific activity of ³²Pi was present as during the initial period of steady-state labeling [*lanes 9–18*]), and the [³⁵S]methionine incorporation was chased by the addition of 10 mM unlabeled methionine [*lanes 1–9*]), and washed and homogenized in the presence of 1 mM sodium phosphate. From one half of the homogenate, total membranes (*T*) were prepared. The other half was fractionated into LDM and PM as described in METHODS.

GLUT4 results in a 3.0- to 5.3-fold increase in the amount of GLUT4 phosphoprotein in total membranes, LDM and PM of desensitized versus normal adipocytes (Table 3). This suggests that insulin resistance of the adipocyte glucose transport *in vitro* is accompanied by significant phosphorylation of GLUT4 in both LDM and PM.

Glimepiride reduces the phosphorylation state of GLUT4 in insulin-resistant adipocytes. Short-term incubation of desensitized adipocytes with both insulin (10 nM) and glimepiride (20 μ M) causes a pronounced decrease of the GLUT4 phosphorylation state in total membranes, LDM and PM (Fig. 8B, lanes 13–18, and 8F). However, glimepiride is much more effective than insulin (Table 3). The ratios in the amount of GLUT4 phosphoprotein in LDM and PM only slightly differ between control and insulin treatment. In contrast, glimepiride treatment causes preferential reduction of the GLUT4 phosphorylation state in LDM versus PM, however, only in desensitized cells (Table 3). Acute treatment of normal adipocytes (which contain only a modest amount of GLUT4 phosphoprotein) with insulin or glimepiride reduces GLUT4 phosphorylation to a similar but minor extent (Fig. 8A, lanes 13–18, and 8E).

In a control experiment (Fig. 9), we tested whether the 32 Pi-labeled and immunoprecipitated 43,000- M_r protein is identical to GLUT4. Cell homogenates from 32 Pi-labeled normal and desensitized adipocytes that have been incubated in the absence or presence of insulin and glimepiride were immunoprecipitated with a polyclonal anti-GLUT4 antibody (raised against the 16 COOH-terminal amino acids of rat GLUT4) or preimmune serum. After SDS-PAGE of the immunoprecipitates, the radiolabeled 43,000- M_r protein bands, identified by autoradiography (Fig. 9, lanes 1–5), were eluted from the gel, separated by SDS-PAGE, and assayed for anti-GLUT4 immunoreactivity by Western blotting with a different polyclonal anti-GLUT4 antibody (raised against purified rat GLUT4) and detection of antibody binding by a chemiluminescence assay. The X-ray film exposed to the nitrocellulose sheet (Fig. 9, lanes 6–10) demonstrates the identity of the phosphorylated and (with anti-GLUT4 antiserum) immunoprecipitated 43,000- M_r protein (Fig. 9, lanes 2–5) with GLUT4 (Fig. 9, lanes 7–10) and thus, together with the nonreactivity of the preimmune serum (Fig. 9, lanes 1 and 6), the specificity of the immunoprecipitation. Furthermore, the experiment confirms that desensitized adipocytes contain less GLUT4 protein than normal adipocytes, which is, however, in a more heavily phosphorylated state (compare Fig. 9 lanes 2 and 3 with lanes 7 and 8). Again, acute glimepiride treatment does not affect the total amount of GLUT4 protein but significantly reduces the amount of GLUT4 phosphoprotein (Fig. 9, lanes 5 and 10). These data suggest that glimepiride decreases the phosphorylation of GLUT4, especially in the LDM fraction of desensitized adipocytes, to a higher degree than insulin.

DISCUSSION

The aim of this study was to elucidate direct effects of sulfonylurea drugs on glucose transport in insulin-resistant isolated rat adipocytes. The insulin resistance was induced by hyperinsulinemic and hyperglycemic conditions in primary culture. The reduced maximal insulin responsiveness and sensitivity of glucose transport in these cells may originate from a postinsulin binding and coupling defect between the insulin receptor and the translocation machinery and synthesis of GLUT molecules, because we observed impaired redistribution of GLUT1 and GLUT4 from LDM to PM and a lowered total number of GLUT1 and GLUT4. Both reduced translocation and synthesis of GLUT4 are characteristic features for adipose tissue of obese and nonobese NIDDM patients (48,49). This suggests similar mechanisms of fat cell insulin resistance operating *in vivo* and *in vitro*. Marshall et al. (50) previously described the effect on translocation but could not find any effect on the total GLUT number. The reason for this discrepancy is not clear but may be related to the different methods used for the identification of GLUT, specific antibodies in our study versus cytochalasin B binding (50).

Effects of glimepiride on glucose transport and GLUT translocation in adipocytes. In insulin-resistant cells, the sulfonylurea drug glimepiride, in the absence of insulin, stimulates glucose transport with only slightly reduced sensitivity and responsiveness compared with normal cells. In fact, a higher maximal initial transport velocity is yielded in insulin-resistant cells by glimepiride than by insulin (Fig. 4A). The glucose transport stimulation may rely at least in part on the increased expression of GLUT1 and GLUT4 at the cell surface in response to drug. This is caused by enhanced translocation rather than increased synthesis of GLUT1 and GLUT4 in both normal and desensitized adipocytes. Thus, this study demonstrates, for the first time, stimulation of GLUT recruitment from LDM to PM by a sulfonylurea drug in the absence of insulin. In addition, these compounds potentiate the insulin-induced redistribution (51).

The molecular site(s) of glimepiride action in adipocytes. The data suggest that glimepiride interacts with the signaling cascade between the insulin receptor and the translocation apparatus downstream of the molecular defect of glucosamine-induced desensitization. The observation that glimepiride present during the primary culture instead of insulin also leads to insulin resistance of the glucose transport provides further evidence for the role of increased glucose influx for desensitization. Interestingly, a glycosyl-phosphatidylinositol-specific phospholipase C becomes activated by glimepiride in cultured 3T3 adipocytes (41) and in normal and insulin-resistant isolated rat adipocytes (G.M., S.W., and A. Korndorfer, unpublished observations). The observation that activation of this phospholipase requires GLUT translocation and experimental evidence from other lab-

Equivalent amounts of protein were solubilized and subjected to immunoprecipitation using anti-GLUT4 peptide antibodies adsorbed to protein A-Sepharose. The collected and washed immunoprecipitates were analyzed by SDS-PAGE and fluorography. The results of one typical experiment repeated 4 times are shown in A and B. The radioactivities contained in the gel bands of four independent experiments were determined by a Berthold scanner. The radiolabel of the immunoprecipitated GLUT4 in total membranes (7) of adipocytes cultured in glucose medium and in the basal state was set at 1.0 arbitrary units. Each value represents the mean of four fractionations \pm SE (C-F).

TABLE 3
 $[^{32}\text{P}]:[^{35}\text{S}]$ ratio of immunoprecipitated GLUT4 in membrane fractions of metabolically labeled normal and desensitized adipocytes after control, acute insulin, and glimepiride treatment calculated from Fig. 8

| | Normal adipocytes | | | Desensitized adipocytes | | |
|----------------|-------------------|---------|-------------|-------------------------|---------|-------------|
| | Control | Insulin | Glimepiride | Control | Insulin | Glimepiride |
| Total membrane | 1 | 0.75 | 0.63 | 5.27 | 4.04 | 2.01 |
| LDM | 1.01 | 0.64 | 0.62 | 5.47 | 4.44 | 1.52 |
| PM | 1.05 | 0.80 | 0.78 | 3.02 | 3.26 | 2.25 |
| LDM:PM | 0.95 | 0.80 | 0.79 | 1.81 | 1.36 | 0.68 |

The last line indicates the ratio of GLUT4 phosphoprotein in LDM and PM.

oratories (52) argue against direct regulation of glucose transport via phospholipase C cleavage products (phosphoinositolglycan headgroups) and suggest a different site of glimepiride action on glucose transport.

Regulation of GLUT4 phosphorylation by insulin and glimepiride. Reports from several laboratories indicate that phosphorylation of GLUT4 at serine/threonine residues by isoproterenol-stimulated PKA (46) or Ca^{2+} /calmodulin-dependent protein kinase (53) or inhibition of protein phosphatases 1 and 2 A by okadaic acid (47,47a) reduces the insulin-stimulated glucose transport in isolated rat adipocytes. Therefore, one possible mechanism for desensitization of the glucose transport and GLUT translocation for insulin stimulation may reside in an increased phosphorylation state of GLUT4. If this is true, activation of glucose transport and GLUT translocation by treatment of desensitized adipocytes with glimepiride should be correlated with diminished GLUT4 phosphorylation. This is exactly what we observed with GLUT4 from both LDM and PM of desensitized adipocytes before and after glimepiride treatment. In contrast, insulin is less effective in reducing GLUT4 phosphorylation. Because extensive GLUT4 phosphorylation in insulin-resistant cells can be observed within 2 h, GLUT4 has to undergo a rapid phosphorylation/dephosphorylation cycle. Its equilibrium is shifted to phosphorylation during desensitization and dephosphorylation during short-term glimepiride and (to a minor degree) insulin treatment.

These modifications may be provoked by stimulation of GLUT4 protein kinases (i.e., PKA, PKC) during desensitization, which are inhibited by glimepiride (and insulin). Alternatively, GLUT4 protein phosphatase(s) may be inhibited during desensitization but stimulated by glimepiride (and insulin). Although kinases/phosphatases acting on GLUT4 in rat adipocytes have not been identified unequivocally so far (54,55), we prefer the former possibility for the following reasons. First, it has been shown in isolated adipocytes that hyperglycemia- and catecholamine-induced insulin resistance are associated with increased PKC and PKA activity, respectively, and decreased insulin receptor kinase (56,57) and glucose transport activity (58). This may be responsible for the downregulation of the insulin signaling cascade. It is possible that similar mechanisms operate during primary culture of rat adipocytes in the presence of high concentrations of insulin and glucose and in NIDDM in the presence of high levels of insulin or counterregulatory hormones that elevate cAMP levels (59). Second, we

were able to demonstrate that incubation of rat adipocytes with glimepiride lowers intracellular cAMP levels via stimulation of cAMP phosphodiesterase and reduces the intrinsic activity of PKA (G.M., S.W., unpublished observations). This has been described previously for other sulfonylureas in rat hepatocytes and adipocytes (60,61). Third, participation of PKA in phosphorylation and desensitization of GLUT4 is suggested by in vitro phosphorylation experiments of immunoprecipitated GLUT4 from normal and desensitized adipocytes with $[^{32}\text{P}]\text{ATP}$ and purified PKA from bovine heart (data not shown). The significantly reduced cAMP-dependent ^{32}P -incorporation into GLUT4 in desensitized versus normal adipocytes indicates that GLUT4 molecules had already been phosphorylated at sites recognized by PKA during the period of desensitization.

Regulation of glucose transport in adipocytes by GLUT phosphorylation. Interestingly, Begum and Draznin (62) previously reported increased GLUT4 phosphorylation in adipocytes from STZ-induced diabetic rats (62). In contrast to normal cells, insulin did not cause GLUT4 dephosphorylation in diabetic adipocytes. The authors presented evidence that the highly phosphorylated GLUT4 in the PM of diabetic adipocytes does not interact with particulate phosphatases. This provides another explanation for the shift in the phosphorylation/dephosphorylation equilibrium of GLUT4 in the diabetic state. However, whereas the GLUT4 phenotype is very similar between their animal model and our in vitro model of desensitized fat cells, the molecular mechanisms for altered GLUT4 phosphorylation may differ between insulin resistance caused by hypo- and hyperinsulinemia.

The correlation between the GLUT4 phosphorylation state and glucose transport velocity in normal, desensitized, and desensitized and subsequently glimepiride-treated rat adipocytes strongly argues for regulation of glucose transport by phosphorylation. Operation of this mechanism during induction of insulin resistance in vitro is also suggested by recent studies of Draznin et al. (63,64) with isolated rat adipocytes incubated with parathyroid hormone (63) or ATP and thapsigargin (64), which both increase cytosolic calcium. These conditions lead to insulin-resistant glucose transport as well as phosphorylation of GLUT4 and reduced insulin-stimulated dephosphorylation of GLUT4. These results strengthen the view that in diverse cellular models of insulin resistance, reduced insulin-stimulated glucose transport is caused

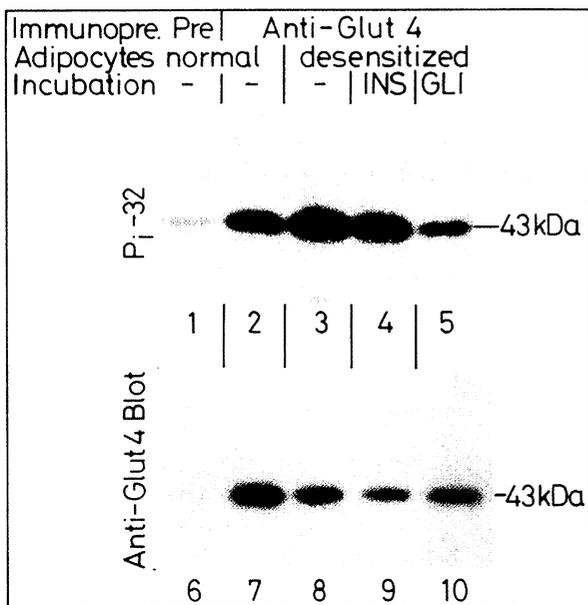


FIG. 9. Specificity of the immunoprecipitation of GLUT4. Adipocytes were cultured in glucose (normal) or glucose/glutamine/insulin medium (desensitized) for 20 h at 37°C, then labeled with ^{32}P i for 2 h at 37°C, and finally incubated in the absence or presence of insulin (10 nM) and glibenclamide (20 μM) for 20 min at 37°C as described for Fig. 8. Cell homogenates were prepared and immunoprecipitated with preimmune serum (Pre) or anti-GLUT4 antiserum adsorbed to protein A-Sepharose. The collected immunoprecipitates were separated by SDS-PAGE and analyzed by autoradiography (lanes 1–5). The protein contained in the radiolabeled 43,000-M_r band was electroeluted from the sliced gel strip, subjected to SDS-PAGE, and transferred to nitrocellulose filter, which was used for immunoblot analysis with a different polyclonal rabbit anti-GLUT4 antibody (raised against total rat GLUT4) using anti-rabbit IgG coupled to alkaline phosphatase and a chemiluminescence assay for detection of antibody binding (lanes 6–10).

by the loss of insulin's ability to elicit dephosphorylation of GLUT4.

However, the effect of GLUT4 phosphorylation on glucose transport may differ between the different *in vitro* models of insulin resistance. The present experimental evidence that phosphorylation of GLUT4 and its cellular distribution between LDM and PM are regulated in parallel, i.e., increased phosphorylation of GLUT4 is correlated with decreased cell surface expression (Fig. 8), suggests that phosphorylation of GLUT4 may act as a signal for its endocytosis (as shown for IGF-II, [65]) or retention in intracellular stores. The observation that glibenclamide treatment preferentially diminishes the phosphorylation state of GLUT4 in LDM (Table 3) favors the possibility that phosphorylation of GLUT4 functions as a retention signal for the intracellular compartment. Interestingly, isolated PM vesicles containing phosphorylated GLUT4 exhibited decreased glucose transport compared with GLUT4 dephosphorylated in response to insulin (63). This raises the possibility that the increased phosphorylation state of GLUT4 interferes with the insulin-stimulated intrinsic glucose transport activity of GLUT4. It remains to be elucidated whether the different underlying mechanisms of glucose transport regulation by phosphorylation, which are not mutually exclusive,

depend on the type of insulin resistance induced *in vitro* and are operating *in vivo*.

Extrapancreatic effects of sulfonylureas *in vitro* and *in vivo*. In addition to adipose tissue, prolonged exposure to high glucose has been reported to acutely and chronically downregulate the glucose transport system in skeletal muscle, which represents the major site for insulin-dependent glucose disposal (66). It will be interesting to see in future studies whether glibenclamide will compensate the lower number of GLUT4 in PM of muscle cells in the basal and insulin-stimulated state. The direct stimulation of glucose transport in the isolated rat diaphragm (67), perfused rat hindlimb (68), and cultured muscle cells (7,8) by sulfonylurea drugs suggests similar modes of action of these compounds in muscle and adipose tissue.

The stimulation of glucose transport by glibenclamide in insulin-resistant adipocytes, which persists even after long-term treatment, may have important implications for the therapy of NIDDM. In contrast to insulin, glibenclamide partially circumvents downregulation of the cell surface GLUT expression by hyperinsulinemia. This feature may contribute to the long-lasting blood glucose-lowering capacity of this drug (23), even after chronic therapy. The physiological significance of the extrapancreatic action of glibenclamide is not weakened by the low total serum levels of glibenclamide (200–400 nM at maximum, E. Draegér, unpublished observations). At this concentration, small but significant increases of glucose transport and GLUT translocation are already observed *in vitro*. The situation may be different when the insulin resistance is induced by hypoinsulinemia instead of hyperinsulinemia because glucose transport in adipocytes isolated from STZ-induced diabetic rats is not stimulated by sulfonylureas in the absence of insulin (15). Furthermore, these drugs have no effect on *in vivo* insulin action in STZ-induced diabetic rats under conditions of severe insulin deficiency. However, in the case of simultaneous administration of insulin using the euglycemic clamp procedure, glibenclamide potentiates insulin-stimulated glucose disposal in peripheral tissues (I. Ohsawa, unpublished observations). Possibly, the expression of a component, which is involved in mediating the glibenclamide signal to the translocation machinery, is under control of insulin and thus missing in the insulinopenic state.

Taken together, this study fits into a speculative framework for a unifying theory of sulfonylurea action based on their capacity to directly stimulate insulin secretion in glucose-insensitive pancreatic β -cells and GLUT translocation in insulin-resistant fat and muscle cells. Both events involve regulated exocytosis and fusion of intracellular vesicles, i.e., secretory granules and insulin-sensitive glucose transporter vesicles budding from tubulo-vesicular structures of the trans-Golgi reticulum (69,70), respectively, with the PM, which may be controlled by similar mechanisms. Sulfonylurea receptors have been described for both rat β -cells (71) and adipocytes (72), but their structural relationship remained unclear. All the other effects of sulfonylurea drugs on glucose metabolism and insulin sensitivity observed on isolated cells and *in vivo* may represent secondary

events following altered glucose influx and blood glucose levels, respectively. In support of this hypothesis, correction of hyperglycemia with phlorizin has been reported to normalize insulin action in diabetic rats (73,74).

ACKNOWLEDGMENTS

The authors wish to thank Dr. E. Draeger (Hoechst AG, Frankfurt) and Dr. W. Bandlow (Genetic Department, University of Munich) for valuable discussions, E.A. Dearey for technical assistance during some experiments, Drs. P. Hale and A. Diedenhofen (Hoechst AG, Frankfurt) for critical reading of the manuscript, and Dr. T. Kreis (EMBL, Heidelberg) and Dr. G.V. Jagow (University of Frankfurt) for kindly providing the antisera.

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