

The Sulfonylurea Glimepiride Regulates Intracellular Routing of the Insulin-Receptor Complexes through Their Interaction with Specific Protein Kinase C Isoforms

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ABSTRACT

Sulfonylureas may stimulate glucose metabolism by protein kinase C (PKC) activation. Because interaction of insulin receptors with PKC plays an important role in controlling the intracellular sorting of the insulin-receptor complex, we investigated the possibility that the sulfonylurea glimepiride may influence intracellular routing of insulin and its receptor through a mechanism involving PKC, and that changes in these processes may be associated with improved insulin action. Using human hepatoma Hep-G2 cells, we found that glimepiride did not affect insulin binding, insulin receptor isoform expression, and insulin-induced receptor internalization. By contrast, glimepiride significantly increased intracellular dissociation of the insulin-receptor complex, degradation of insulin, recycling of internalized insulin receptors, release of internalized radioactivity, and prevented insulin-induced receptor down-regulation. Association

of PKC- β II and $-\epsilon$ with insulin receptors was increased in glimepiride-treated cells. Selective depletion of cellular PKC- β II and $-\epsilon$ by exposure to 12-O-tetradecanoylphorbol-13-acetate (TPA) or treatment of cells with PKC- β II inhibitor G06976 reversed the effect of glimepiride on intracellular insulin-receptor processing. Glimepiride increased the effects of insulin on glucose incorporation into glycogen by enhancing both sensitivity and maximal efficacy of insulin. Exposing cells to TPA or G06976 inhibitor reversed these effects. Results indicate that glimepiride increases intracellular sorting of the insulin-receptor complex toward the degradative route, which is associated with both an increased association of the insulin receptor with PKCs and improved insulin action. These data suggest a novel mechanism of action of sulfonylurea, which may have a therapeutic impact on the treatment of type 2 diabetes.

Sulfonylurea drugs have been widely used in the therapy of type 2 diabetes since the discovery of their hypoglycemic effects more than 40 years ago. The hypoglycemic action of sulfonylureas has been attributed primarily to acute stimulation of insulin secretion by pancreas (Lebovitz, 1984). Additional extrapancreatic actions of sulfonylureas on glucose metabolism have been first suggested by the observation that chronic sulfonylurea treatment of patients with type 2 diabetes results in improved glucose tolerance in the absence of elevated insulin levels (Kolterman et al., 1984). Further in vivo studies in humans (Simonson et al., 1984) and animals (Hirshman and Horton, 1990) have documented improvement in glucose tolerance associated with an improvement in insulin sensitivity. A number of in vitro studies have confirmed that sulfonylureas directly stimulate glucose metab-

olism and increase insulin sensitivity in target tissue of insulin action (Rogers et al., 1987; Muller and Wied, 1993; Tsiani et al., 1995).

The concentration of insulin in plasma is the result of both its rate of secretion from pancreatic β -cells and its rate of clearance from the plasma. Receptor-mediated insulin endocytosis is the main mechanism for insulin clearance from the circulation, and liver is a major site of insulin degradation in vivo. After binding of insulin to its receptor located on the cell surface, the hormone-receptor complex is internalized through the formation of clathrin-coated vesicles, and is delivered to endosomes (Carpentier, 1994). Acidification of endosomes allows the dissociation of insulin from its receptor and their sorting in different directions. Most of the internalized hormone is targeted to lysosomes where it is degraded to low-molecular-mass products, whereas a smaller fraction remains intact. Both degradation products and intact insulin are segregated in recycling vesicles and released from cell

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ABBREVIATIONS: PKC, protein kinase C; PAGE, polyacrylamide gel electrophoresis; IRS, insulin receptor substrate; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; TCA, trichloroacetic acid; PEG, polyethylene glycol; TPA, 12-O-tetradecanoylphorbol-13-acetate.

(Marshall, 1985a; Levy and Olefsky, 1987). By contrast, most of the internalized receptor is recycled to the cell surface to be reutilized, and only a small fraction is degraded (Marshall, 1985b). Several lines of evidence suggest that internalization and intracellular processing of the insulin-receptor complex may play a role in insulin action (Peavy et al., 1984; Veda et al., 1985; Jochen and Berhanu, 1987; Miller, 1988). Association between impaired *in vivo* insulin clearance and action have been reported in subjects with insulin resistance (Ferrannini et al., 1982; Flier et al., 1982). Moreover, defects in insulin-receptor internalization and processing have been reported in various cells from patients with type 2 diabetes mellitus including circulating monocytes (Grunberger et al., 1989; Trischitta et al., 1989; Benzi et al., 1990; Benzi et al., 1997), isolated adipocytes (Jochen et al., 1986) and cultured Epstein Barr virus-transformed lymphocytes (Sesti et al., 1996), thus suggesting that abnormalities of these processes might contribute to the insulin resistance of type 2 diabetes. Overall, these findings raise the possibility that a sulfonylurea-induced improvement in intracellular insulin degradation may explain the decrease in plasma insulin levels associated with improved glucose tolerance observed in patients with type 2 diabetes chronically treated with sulfonylureas (Kolterman et al., 1984). The molecular mechanism by which sulfonylureas act remains unclear, although most of the available data suggest that sulfonylureas act at postreceptor level (Jacobs et al., 1987; Bak et al., 1989). Some evidence suggests that sulfonylureas stimulate glucose transport through a mechanism involving protein kinase C (PKC) activation (Cooper et al., 1990). Evidence is also available showing that interaction of the insulin receptor with PKC plays an important role in controlling the intracellular sorting of the insulin-receptor complex toward the degradative route (Formisano et al., 1998). We therefore inquired whether sulfonylureas might influence intracellular routing of insulin and its receptor through a mechanism involving PKC and whether changes in these processes are associated with an improvement in the metabolic action of insulin. To this end, we have used the human hepatoma cell line Hep-G2 to examine the effects of glimepiride, a novel sulfonylurea drug, on insulin-induced receptor internalization and recycling, and intracellular insulin degradation. The results show that glimepiride increased intracellular sorting of the insulin-receptor complex toward the degradative route that was associated with both an increased association of the insulin receptor with PKC and improved insulin responsiveness and sensitivity for glucose incorporation into glycogen. These data suggest a novel mechanism of action of sulfonylurea, which may have a therapeutic impact on the treatment of type 2 diabetes.

Experimental Procedures

Materials. ^{125}I -Tyr^{A14}-monoiodoinsulin (300–350 $\mu\text{Ci}/\mu\text{g}$) was purchased from Amersham Pharmacia Biotech (Milano, Italy). The IgG fraction (I-2 IgG) of the human antiserum, which recognizes exclusively the Ex11⁺ insulin receptor isoform (Ullrich et al., 1985) was isolated by affinity chromatography on a protein-A Sepharose CL4B column, as described previously (Sesti et al., 1992, 1994a). An anti-insulin receptor polyclonal antibody to the COOH-terminus of the β -subunit was produced as described previously (Sesti et al., 1994b). Antiphosphotyrosine polyclonal antibody was from Transduction Laboratories (Lexington, KY). Polyclonal antibodies directed

toward specific PKC isoforms were from Life Technologies (Grand Island, NY). A polyclonal anti-PKC- β II antibody was from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). PKC- β II inhibitor G06976 was purchased from Calbiochem (La Jolla, CA). SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot reagents were from Bio-Rad (Richmond, CA). Anti-IRS-1 and anti-IRS-2 polyclonal antibodies were purchased from UBI (Lake Placid, NY). Enhanced chemiluminescence reagent detection system (SuperSignal CL-HRP Substrate System) was from Pierce (Rockford, IL). All other chemicals were from Sigma Chemicals Co. (St. Louis, MO).

^{125}I -Insulin Binding and Quantification of Relative Abundance of the Two Receptor Isoforms. ^{125}I -insulin binding studies and measurements of relative abundance of the two insulin receptor protein isoforms were performed as described previously (Sesti et al., 1992, 1994a). Briefly, Hep-G2 cells were cultured in the presence or absence of various concentrations of glimepiride for the indicated periods of time. Thereafter, cells were rinsed twice with DMEM containing 1% bovine serum albumin (BSA), and incubated with ^{125}I -insulin (50 pmol/l) for 16 h at 4°C in the presence or absence of increasing concentrations of unlabeled insulin or I-2 IgG. Bound radioactivity was determined by washing cells twice with ice-cold PBS, and scraping cells from wells with 0.03% SDS. Quantification of the two insulin receptor mRNA splicing isoforms by reverse transcription reaction, followed by polymerase chain reaction, was performed according to methods described previously (Sesti et al., 1994a).

Insulin Receptor and IRS-1/IRS-2 Tyrosine Phosphorylation. Hep-G2 cells cultured in the presence or absence of glimepiride for 72 h were rinsed twice with DMEM/1% BSA and incubated with 100 nM insulin for the indicated periods of time at 37°C. At the end of incubation, cells were washed with ice-cold PBS and lysed for 45 min at 4°C in lysis buffer containing 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 2 mM EDTA, 10 mM NaPP₆, 2 mM sodium orthovanadate, 10 mM NaF, 8 $\mu\text{g}/\text{ml}$ leupeptin, 2 mM phenylmethylsulfonyl fluoride, 2 mM Na₃VO₄, 10% glycerol, and 1.5% Nonidet P-40. Insoluble material was removed by centrifugation in Microfuge for 10 min, and the supernatant was saved. Equal amounts of cell lysates were incubated for 16 h at 4°C with 5 μg of anti-insulin receptor, anti-IRS-1 antibody, or anti-IRS-2 antibody. Immune complexes were collected by incubation with protein A-Sepharose for 2 h at 4°C, and equal amounts of immunoprecipitated proteins were subjected to SDS-PAGE under reducing conditions. Proteins resolved by SDS-PAGE were electrophoretically transferred to nitrocellulose membrane. The nonspecific binding sites of membranes were blocked by a 2 h incubation in 10 mM Tris, pH 7.5, and 150 mM NaCl buffer with 0.1% Tween 20 and 1% BSA. The membranes were then incubated for 1 h at room temperature with peroxidase-conjugated antiphosphotyrosine antibody. Proteins were detected by using enhanced chemiluminescence, and band densities were quantified by densitometry using a Fluor-S MultiImager (Bio-Rad).

Insulin-Receptor Complex Internalization—Single Cohort Method. Hep-G2 cells cultured in the presence or absence of glimepiride for the indicated periods of time were washed twice with DMEM containing 1% BSA, and incubated for 4 h at 4°C in the presence of ^{125}I -insulin (50 pmol/l). Unbound ^{125}I -insulin was then removed by three additional washes with cold DMEM. Cells were then rapidly warmed by the addition of DMEM/1% BSA at 37°C and incubated at 37°C for the indicated periods of time. Thereafter, cells were acid washed with sodium acetate buffer, pH 3, for 5 min at 4°C to determine the fraction of internalized radioactivity or were washed twice with ice-cold PBS to determine total cell-associated radioactivity. Cells were then solubilized with 0.03% SDS and radioactivity was counted.

Insulin-Induced Receptor Internalization and Recycling. Hep-G2 cells were cultured in the presence or absence of various concentrations of glimepiride for the indicated periods of time. Thereafter, cells were rinsed twice with DMEM/1% BSA, and incubated in the presence or absence of 1 nmol/l unlabeled insulin for the

periods of time (indicated under *Results*) at 37°C. Cells were then washed at 4°C to remove unbound insulin and then acid washed with sodium acetate buffer, pH 4.5, for 15 min at 4°C to dissociate cell-surface-bound insulin. ^{125}I -insulin (50 pmol/l) was added to cells and insulin binding to residual cell-surface receptors was performed for 16 h at 4°C. Under these conditions of incubation, insulin internalization and receptor recycling are negligible. The recovery of cell-surface insulin binding after receptor internalization (receptor recycling) was studied by incubating Hep-G2 cells in the presence or absence of 1 nmol/l unlabeled insulin for 30 min at 37°C. Acid washed cells were then incubated in DMEM/1% BSA for 30 min at 37°C in the absence of insulin to allow the recovery of cell-surface insulin binding. Thereafter, ^{125}I -insulin (50 pmol/l) was added to cells and insulin binding to cell-surface receptors was carried out for 16 h at 4°C. Bound radioactivity was determined by washing cells twice with PBS, and solubilizing cells with 0.03% SDS. In all experiments, nonspecific binding, defined as the binding in the presence of 1 $\mu\text{mol/l}$ unlabeled insulin, was subtracted, and it was always less than 10% of total binding.

Release of Internalized Radioactivity. Hep-G2 cells cultured in the presence or absence of glimepiride for 72 h were rinsed twice with DMEM/1% BSA and incubated with 600 pmol/l of ^{125}I -insulin for 60 min at 37°C to reach maximum insulin internalization. Thereafter, cells were acid washed for 15 min at 4°C to remove ^{125}I -insulin bound to cell-surface receptors, and incubated in DMEM/1% BSA for the indicated periods of time at 37°C. The amount of residual intracellular radioactivity was determined by washing cells twice and solubilizing cells with 0.03% SDS. The nature of the radioactivity released by cells in the incubation medium was analyzed by both trichloroacetic acid (TCA) precipitability and Sephadex G-50 column chromatography.

Dissociation of the Internalized Insulin-Receptor Complex. The proportion of the intracellular ^{125}I -insulin that remained bound to the receptor was determined by the polyethylene glycol (PEG) assay previously described (Levy and Olefsky, 1988; Sesti et al., 1996). Using this method, PEG-precipitable radioactivity represents ^{125}I -insulin bound to the receptor, whereas PEG-soluble material represents radioactivity that had dissociated from the internalized receptor.

Association of the Insulin Receptor with PKC. Hep-G2 cells cultured in the presence or absence of glimepiride for 72 h were rinsed twice with DMEM/1% BSA and incubated with 100 nM insulin for the indicated periods of time at 37°C. At the end of incubation, cells were washed with ice-cold PBS and lysed for 30 min at 4°C in lysis buffer. Insoluble material was removed by centrifugation, and cell lysates were incubated for 16 h at 4°C with 1 μg anti-insulin receptor antibody followed by incubation with protein A-Sepharose for 2 h at 4°C. Equal amounts of immunoprecipitated proteins were subjected to SDS-PAGE under reducing conditions, and electrophoretically transferred to nitrocellulose membranes. The membranes were then incubated for 16 h at 4°C with isoform-specific PKC antibodies. After extensive washings, the blots were incubated with peroxidase-conjugated goat anti-rabbit IgG antibodies. Proteins were detected by using enhanced chemiluminescence, and band densities were quantified by densitometry.

Glucose Incorporation into Glycogen. Hep-G2 cells cultured in the presence or absence of glimepiride for 72 h were rinsed twice with DMEM/1% BSA and incubated with the same medium containing 25 mM HEPES, pH 7.6, and glucose (final concentration, 2.5 mM) for 3 h at 37°C. Insulin at the indicated concentrations and [^{14}C]glucose (4 μCi) were then added to each well followed by a 2-h incubation. Wells were washed with PBS, and cells were solubilized in 0.5 ml 30% KOH containing 2 mg of unlabeled glycogen and incubated for 30 min at 37°C. The mixture was boiled for 15 min and glycogen was precipitated in 70% ethanol on ice. The precipitate was pelleted by centrifugation, washed with 70% ethanol, and dissolved in water. Radioactivity was determined by scintillation counting.

Statistical Analysis. All data are expressed as the mean \pm S.D. and analyzed statistically by unpaired Student's *t* test. When appropriate, a two-way analysis of variance test was used to compare data from the cell lines.

Results

Insulin Binding, Relative Abundance of the Two Insulin Receptor Isoforms, and Insulin-Induced Receptor and IRS-1/IRS-2 Tyrosine Phosphorylation. Insulin binding and relative abundance of the two insulin receptor isoforms were studied in Hep-G2 cells cultured for 72 h in the presence or absence of 20 $\mu\text{mol/l}$ glimepiride. The displacement of tracer ^{125}I -insulin by increasing concentration of unlabeled insulin was similar in glimepiride-treated or -untreated cells, with EC_{50} values for insulin binding occurring at 0.3 to 0.4 nmol/l. At steady state, maximal ^{125}I -insulin binding did not differ between glimepiride-treated cells and glimepiride-untreated cells [B/T (^{125}I -insulin bound versus total added radioactivity) = 12 ± 3 and $11 \pm 3\%$, respectively]. The relative abundance of the two insulin receptor isoforms (Ex11^- and Ex11^+) was measured by an immunoassay validated previously (Sesti et al., 1992, 1994a). This assay is based upon the ability of a human anti-receptor autoantibody (I-2 IgG) to inhibit ^{125}I -insulin binding to the Ex11^- receptor isoform expressed on cell surface without affecting insulin binding to the Ex11^+ isoform (Sesti et al., 1992, 1994a). Maximally effective concentration of I-2 IgG (2 $\mu\text{mol/l}$) inhibited ^{125}I -insulin binding to glimepiride-treated cells by $53 \pm 3\%$ and to untreated cells by $57 \pm 6\%$. Same results were obtained when a polymerase chain reaction-based assay was used to determine the relative abundance of the two receptor mRNA transcripts (data not shown). Therefore, no significant differences were found in total cell-associated radioactivity, insulin binding affinity, and relative abundance of insulin receptor isoforms in glimepiride-treated cells compared with untreated cells. To investigate whether glimepiride affects postbinding insulin effects, insulin-induced receptor phosphorylation was determined. As shown in Fig. 1, insulin stimulated tyrosine phosphorylation of its receptor to the same extent in both glimepiride-treated and

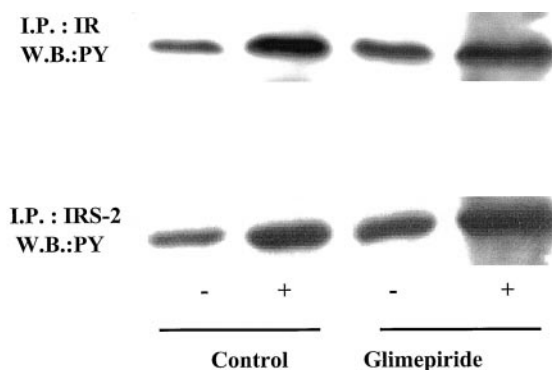


Fig. 1. Tyrosine phosphorylation of the insulin receptor and IRS-2 in response to insulin. Hep-G2 cells cultured in the absence or presence of 20 $\mu\text{mol/l}$ glimepiride for 72 h were exposed to 100 nM insulin for 5 min. The cells were lysed and equal amounts of proteins were immunoprecipitated with anti-insulin receptor (top) or anti-IRS-2 (bottom) antibody, separated by SDS-PAGE, transferred to nitrocellulose membrane, and Western immunoblotted with anti-phosphotyrosine antibody. Proteins were detected by using enhanced chemiluminescence, and band densities were quantified by densitometry. The autoradiographs shown are representative of three independent experiments.

-untreated cells. Some evidence suggests that IRS-2 is the main effector of both metabolic and mitogenic actions of insulin in hepatocytes (Rother et al., 1998). We therefore inquired whether glimepiride affects the ability of insulin to activate IRS-2. As shown in Fig. 1, tyrosine phosphorylation of IRS-2 in response to insulin was similar in both glimepiride-treated and untreated cells. In addition, insulin-stimulated tyrosine phosphorylation of IRS-1 did not differ between glimepiride-treated and -untreated cells (data not shown).

Insulin-Receptor Complex Internalization. As shown in Fig. 2, both glimepiride-treated and -untreated cells rapidly internalize the cell surface bound ^{125}I -insulin; maximal effect occurs after 20 min of incubation at 37°C. Preincubation of cells with glimepiride for various periods of time did not affect either the rates of ^{125}I -insulin internalization or the amount of internalized ^{125}I -insulin.

Insulin-Induced Receptor Internalization and Receptor Recycling. The time course of insulin-induced receptor internalization was studied in Hep-G2 cells cultured for 72 h in the presence or absence of 20 $\mu\text{mol/l}$ glimepiride, and exposed to 1 nmol/l native insulin for 30 min at 37°C. Previous studies have shown that under these conditions, insulin receptors are internalized through an endocytotic pathway involving coated-pits and endosomal acidification (Levy and Olefsky, 1987; McClain and Olefsky, 1988). After exposure to insulin, a significant reduction of the subsequent cell-surface ^{125}I -insulin binding was seen within 5 min, and apparent steady state levels were reached after 20 min in both glimepiride-treated and -untreated cells. When cells were incubated with insulin at 4°C, insulin-induced receptor internalization was <3% in both cases. Exposure of cells to 1 nmol/l insulin for 30 min reduced subsequent cell-surface ^{125}I -insulin binding to a similar extent in control and glimepiride-treated cells (59 ± 2 versus $60 \pm 4\%$ of initial binding values, respectively). By contrast, the recovery of initial ^{125}I -insulin binding was significantly higher in cells treated with glimepiride than in control cells (111 ± 6 versus $90 \pm 2\%$, $P < 0.006$, respectively). Time-course studies revealed that glimepiride produces a significant effect on insulin receptor recycling by 24 h, with maximal effect occurring by 48 h and remaining constant for 72 h. The dose-dependent

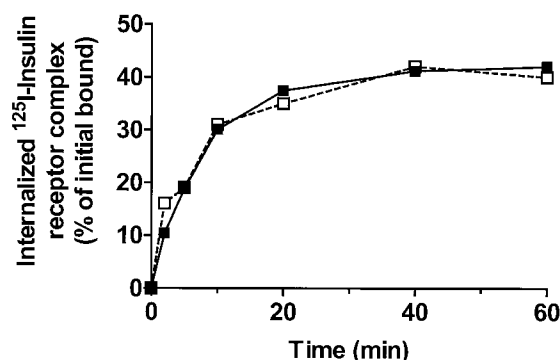


Fig. 2. Internalization of a single cohort of surface-bound insulin. Hep-G2 cells cultured in the absence (□) or presence (■) of 20 $\mu\text{mol/l}$ glimepiride were washed, and incubated for 4 h at 4°C in the presence of ^{125}I -insulin (50 pmol/l). After removal of unbound insulin, cells were rapidly warmed to 37°C. At the indicated periods of time, the fraction of radioactivity internalized by cells was measured after acid-washing. Results of a representative experiment carried out in triplicate are expressed as the percentage of total ^{125}I -insulin bound at time 0.

analysis of glimepiride effect revealed a maximal action at 20 $\mu\text{mol/l}$, with half-maximal effect occurring at ~ 0.5 $\mu\text{mol/l}$, which is slightly higher than the therapeutic concentration usually measured in the serum of patients with type 2 diabetes (0.2–0.4 $\mu\text{mol/l}$).

Release of Intracellular Radioactivity. As shown in Fig. 3, control cells released the intracellular radioactivity in a time-dependent manner. Of the internalized radioactivity, 50% ($t_{1/2}$) was released from cells by 22.5 min, and about 40% remained after 30 min. At each time studied, the rates of loss of internalized radioactivity were significantly increased in Hep-G2 cells cultured in the presence of glimepiride (Fig. 3). Therefore, the $t_{1/2}$ of the release of internalized radioactivity was significantly lower in glimepiride-treated cells than in control cells ($t_{1/2} = 11 \pm 2$ versus 22.5 ± 3 min, $P < 0.03$, respectively). Chromatography analysis of released radioactivity revealed three peaks (Fig. 4A). The first peak eluted with the void volume and represents high-molecular-mass material that is still incompletely characterized. The second peak coeluted with intact ^{125}I -A¹⁴-moniodoinsulin, whereas the third peak, which coeluted with the salt volume, represents low-molecular-mass degradation products. Analysis of material released from cells after 15 min of incubation, revealed that the percentage of total released radioactivity coeluting with intact insulin (peak II) was significantly higher in control cells than in glimepiride-treated cells (48 ± 5 and $33 \pm 5\%$, $P < 0.01$, respectively) (Fig. 4). After 15 min of incubation, the amounts of TCA-precipitable radioactivity (intact insulin) released by cells was significantly higher in control cells than in glimepiride-treated cells (50 ± 4 and $39 \pm 6\%$, $P < 0.01$, respectively). These findings suggest that cells treated with glimepiride degrade insulin more effectively than control cells.

Dissociation of the Internalized Insulin-Receptor Complex and Receptor Down-Regulation. Because dissociation of insulin from its receptor is a prerequisite for both the recycling of the receptor to the cell-surface and the release of insulin from cells, an increase in the dissociation of the insulin-receptor complex may account for the effects of glimepiride on receptor recycling and insulin release. To test this hypothesis, the internalized ^{125}I -insulin remained bound

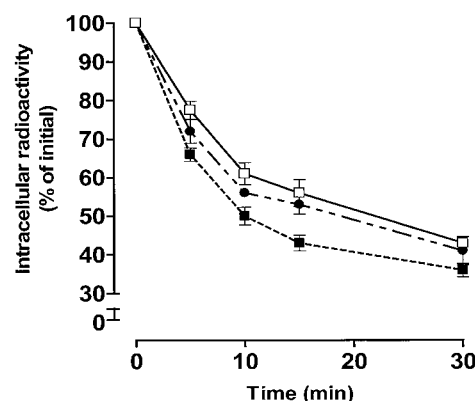


Fig. 3. Rate of release of internalized radioactivity. Hep-G2 cells cultured in the absence (open symbols) or presence (closed symbols) of 20 $\mu\text{mol/l}$ glimepiride for 72 h were preincubated with (●) or without (□, ■) 1 μM TPA followed by incubation with 600 pmol/l ^{125}I insulin for 60 min at 37°C. Cells were acid washed, and incubated for the indicated periods of time at 37°C. Then, the amount of residual intracellular radioactivity was counted. Values are means \pm S.D. of four experiments carried out in triplicate.

to the receptor was determined on the basis of its ability to precipitate in 25% PEG. Figure 5 shows the time course of the percentage of total intracellular radioactivity that was PEG-precipitable. The rate of intracellular dissociation was rapid, with maximal effect occurring after 20 min and remaining relatively constant up to 30 min. At each time studied, the extent of intracellular dissociation were increased in glimepiride-treated cells compared with control cells. After 20 min of incubation, when the dissociation of insulin-receptor complexes was maximal, 33% of the internalized radioactivity was PEG-precipitable with glimepiride-treated cells, thus indicating that 67% of the internalized insulin had dissociated from the receptor. In contrast, with control cells 42% of internalized radioactivity was PEG-precipitable, thus suggesting that a higher proportion of the internalized insulin remains bound to the receptor. An increased insulin receptor recycling without concomitant changes in receptor internalization would be expected to affect insulin-induced receptor down-regulation. To investigate this possibility, Hep-G2 cells cultured in the presence or absence of 20 $\mu\text{mol/l}$ glimepiride for 72 h were exposed to 100 nmol/l insulin for 8 h. As shown in Fig. 6, insulin-induced receptor down-regulation was significantly reduced in cells treated with glimepiride compared with control cells, thus indicating that glimepiride prevents receptor down-regulation during chronic insulin stimulation, presumably by increasing the rate of receptor recycling.

Association of the Insulin Receptor with PKC. There is evidence suggesting that PKC plays an important role in regulating the internalization and intracellular degradation of several tyrosine kinase receptors, including the insulin receptor (Seedorf et al., 1995; Formisano et al., 1998). We therefore inquired whether the effect of glimepiride on the intracellular processing of the insulin-receptor complex was associated with changes in interaction between the insulin receptor and PKC. It has been reported that Hep-G2 cells express four PKC isoforms (α , β , ϵ , and ζ) (Ducher et al.,

1995). We therefore tested the ability of these PKC isoforms to coimmunoprecipitate with the insulin receptor in Hep-G2 cells cultured in the presence or absence of 20 $\mu\text{mol/l}$ glimepiride for 72 h. Under basal conditions, PKC- β and - ϵ were coprecipitated with the insulin receptor in cells cultured in absence of glimepiride (Figs. 7 and 8). Upon stimulation of the cells with 100 nM insulin for 15 min, the amounts of PKC- β II and - ϵ detected in insulin-receptor immunoprecipitates increased by 3.0-fold and 5.3-fold, respectively (Figs. 7 and 8). By contrast, PKC- α , and - ζ could not be detected in insulin receptor immunoprecipitates in either basal or insulin-stimulated cells (data not shown). In Hep-G2 cells cultured with glimepiride, the amounts of both PKC- β II and - ϵ coprecipitated with the insulin receptor under basal condition were increased by 3.6-fold and 3.5-fold, respectively, compared with glimepiride-untreated cells (Figs. 7 and 8). Upon stimulation of glimepiride-treated cells with 100 nM insulin for 15 min, the amounts of PKC- β II and - ϵ coprecipitated with the insulin receptor increased by 2.4-fold and 1.7-fold, respectively, compared with glimepiride-untreated cells (Figs. 7 and 8). Time course of the insulin effect on insulin receptor-PKC coprecipitation was also affected by glimepiride. In cells cultured in the absence of glimepiride, the insulin effect was maximal after 15 min of exposure followed by a decline by 30 min. By contrast, in cells cultured in the presence of glimepiride, a considerable increase was observed after 5 min of exposure that reached a maximum after 30 min (Figs. 7 and 8). Total PKC- β II and - ϵ content was not affected by treatment with glimepiride (Figs. 7 and 8, top).

Effects of PKC Inhibition and Cellular Depletion on Intracellular Processing of the Insulin-Insulin Receptor Complex. To address the question of whether the increased association of the insulin receptor with PKC- β II and - ϵ induced by glimepiride was involved in intracellular processing of the insulin-receptor complex, we analyzed insulin receptor recycling, release of intracellular insulin and its

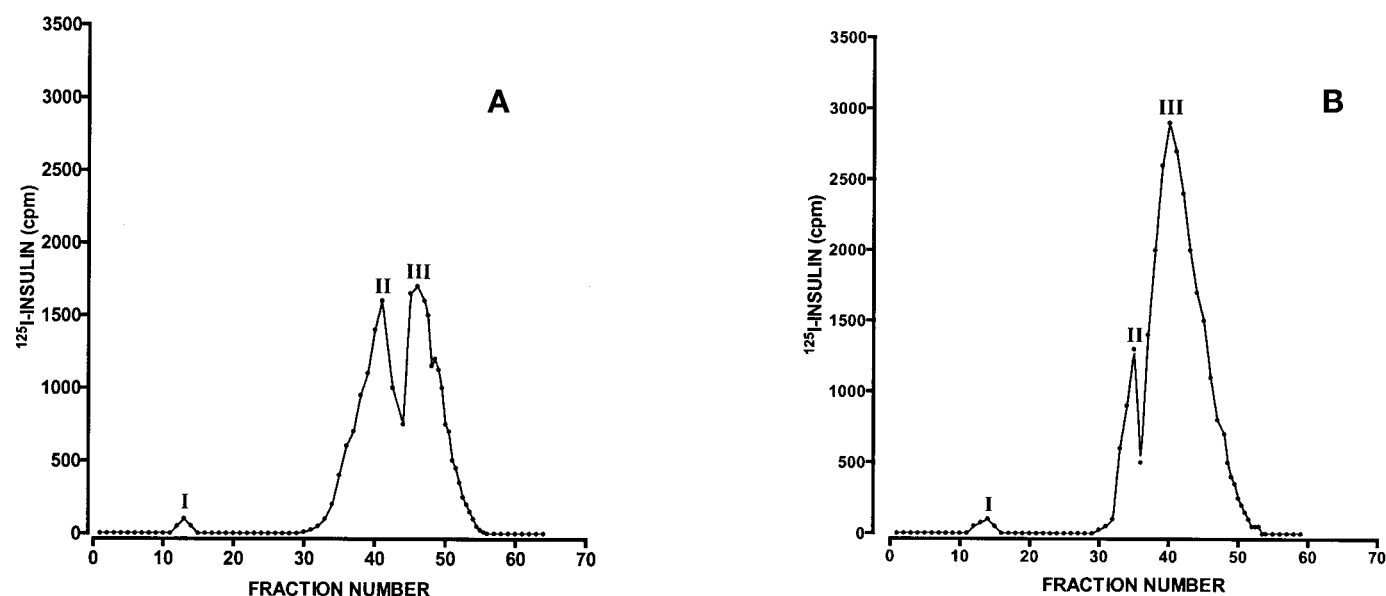


Fig. 4. Sephadex G-50 analysis of internalized radioactivity released in medium by Hep-G2 cells. Cells cultured in the absence (A) or presence (B) of 20 $\mu\text{mol/l}$ glimepiride for 72 h were incubated with 600 pmol/l ^{125}I insulin for 60 min at 37°C, acid washed, and then resuspended at 37°C to allow the release of intracellular radioactivity. After 60 min, the supernatants were applied to a Sephadex G-50 column and eluted. 1 ml fractions were collected and counted. The data are representative of one of three independent experiments.

degradation products after cell depletion of TPA-sensitive PKCs (PKC- β II and - ϵ) or inhibition of PKC- β II with G06976. As expected, treatment of cells with 1 μ M TPA almost completely abolished the amount of PKC- β II coprecipitated with the insulin receptor (Fig. 9). Furthermore, treatment of cells with 1 μ M TPA reduced by 60% expression of PKC- ϵ and decreased by 70% the amount of PKC- ϵ coprecipitated with the insulin receptor compared with glimepiride-treated cells (Fig. 9). To determine whether insulin-induced PKC activation is required for its association with the insulin receptor, and to test the specificity of the PKC- β II antibody used, the cells were incubated in the presence or absence of PKC- β II inhibitor G06976, lysed, immunoprecipitated with anti-insulin antibody, and immunoblotted with a different PKC- β II antibody (Santa Cruz). Treatment of cells with PKC- β II inhibitor G06976 decreased the amount of PKC- β II coprecipitated with the insulin receptor in response to insulin to the level observed in control cells (Fig. 10), thus suggesting that activation of PKC- β II is necessary for its interaction with the insulin receptor. Preincubation with TPA of glimepiride-treated cells almost completely reversed the effect of glimepiride to increase insulin receptor recycling and to accelerate the release of intracellular insulin. The recovery of initial 125 I-insulin binding was significantly decreased in

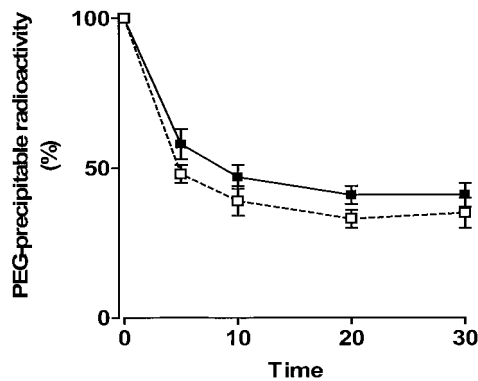


Fig. 5. Time course of the percentage of intracellular radioactivity PEG-precipitable. Hep-G2 cells cultured in the absence (\square) or presence (\blacksquare) of 20 μ M glimepiride for 72 h were incubated with 600 pmol/l of 125 I-insulin for 16 h at 4°C. Internalization was initiated in a synchronous manner by incubating cells at 37°C with prewarmed buffer. At the indicated times, cells were acid washed and solubilized. The remaining intracellular radioactivity was analyzed for its ability to precipitate in 25% PEG. Data are expressed as the percentage of PEG-precipitable radioactivity versus total intracellular radioactivity. Values are means \pm S.D. of six experiments carried out in triplicate.

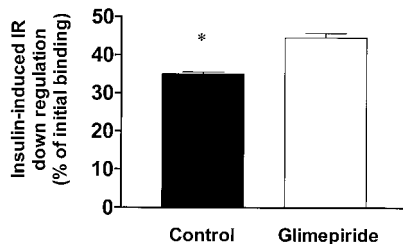


Fig. 6. Insulin-induced receptor down-regulation. Hep-G2 cells cultured in the presence or absence of 20 μ M glimepiride for 72 h were exposed to 100 nmol/l insulin for 8 h at 37°C. Cells were then washed at 4°C to remove unbound insulin and then acid washed for 15 min at 4°C to dissociate cell-surface-bound insulin. 125 I-insulin (50 pmol/l) was added to cells and insulin binding to residual cell-surface receptors was performed for 16 h at 4°C. Values are means \pm S.D. of four experiments carried out in triplicate. * p < 0.012.

cells treated with TPA + glimepiride compared with cells treated with glimepiride alone (90 ± 2 versus $111 \pm 6\%$, P < 0.006, respectively). Similar results were obtained when cells were preincubated with G06976 (96 ± 1.5 versus $111 \pm 6\%$ of initial binding values, P < 0.001, in G06976-treated and -untreated cells, respectively). The rate of loss of internalized radioactivity was significantly decreased in cells treated with TPA at each time studied ($t_{1/2} = 11 \pm 2$ and 19 ± 3 min, P < 0.03, in TPA-untreated cells and TPA-treated cells, respectively) (Fig. 3). Analysis of material released from cells after 30 min of incubation revealed that the amount of TCA precipitable radioactivity (intact insulin) was significantly higher in TPA-treated cells than in TPA-untreated cells (29 ± 1 and $21 \pm 1\%$, P < 0.01, respectively), thus indicating that treatment with TPA reversed the effect of glimepiride to

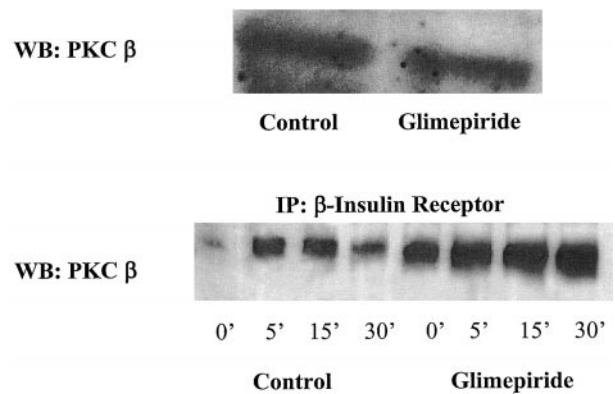


Fig. 7. Coprecipitation of the insulin receptor with PKC- β II isoform. Hep-G2 cells cultured in the absence or presence of 20 μ M glimepiride for 72 h were exposed to 100 nM insulin for the indicated periods of time. The cells were lysed and equal amounts of proteins were immunoprecipitated with anti-insulin receptor antibody, separated by SDS-PAGE, transferred to nitrocellulose membrane, and Western immunoblotted with specific PKC- β II antibody. Equal aliquots of the cell lysates were directly resolved by SDS-PAGE and Western immunoblotted with no previous precipitation (total lysates). Proteins were detected by using enhanced chemiluminescence, and band densities were quantified by densitometry. The autoradiographs shown are representative of three independent experiments.

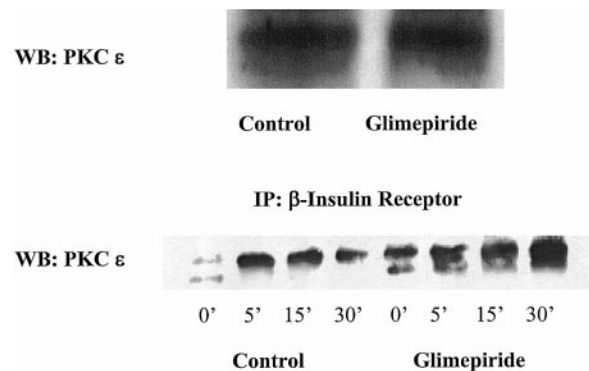


Fig. 8. Coprecipitation of the insulin receptor with PKC- ϵ isoform. Hep-G2 cells cultured in the absence or presence of 20 μ M glimepiride for 72 h were exposed to 100 nM insulin for the indicated periods of time. The cells were lysed and equal amounts of proteins were immunoprecipitated with anti-insulin receptor antibody, separated by SDS-PAGE, transferred to nitrocellulose membrane, and Western immunoblotted with specific PKC- ϵ antibody. Equal aliquots of the cell lysates were directly resolved by SDS-PAGE and Western immunoblotted with no previous precipitation (total lysates). Proteins were detected by using enhanced chemiluminescence, and band densities were quantified by densitometry. The autoradiographs shown are representative of three independent experiments.

promote intracellular insulin degradation. Similarly, after G06976 preincubation, the amount of TCA-precipitable radioactivity (intact insulin) released from cells was significantly higher in G06976-treated cells than in untreated cells (26 ± 0.3 and $21 \pm 0.5\%$, $P < 0.002$, respectively). These findings suggest that glimepiride-induced PKC-insulin receptor association is required to route the insulin-receptor complexes toward the degradative compartment.

Effect of Glimepiride on Insulin-Stimulated Glucose Incorporation Into Glycogen. To investigate whether the effects of glimepiride on insulin-receptor complex processing were associated with changes in insulin action, insulin-stimulated glucose incorporation into glycogen was measured. As shown in Fig. 11, insulin increased glucose incorporation into

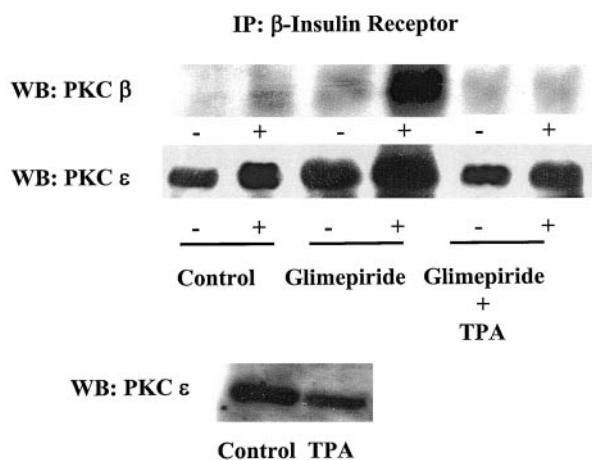


Fig. 9. Coprecipitation of the insulin receptor with PKC isoforms after TPA-induced depletion. Hep-G2 cells cultured in the absence or presence of $20 \mu\text{mol/l}$ glimepiride for 72 were preincubated for 24 h with $1 \mu\text{M}$ TPA, and further incubated in the presence or absence of 100 nM insulin for 30 min. The cells were lysed and equal amounts of proteins were immunoprecipitated with anti-insulin receptor antibody, separated by SDS-PAGE, transferred to nitrocellulose membrane, and Western immunoblotted with specific PKC- β II and - ϵ antibodies (top). Equal aliquots of total cell lysates were directly resolved by SDS-PAGE and Western immunoblotted with specific PKC- ϵ antibody with no previous precipitation (bottom). Proteins were detected by using enhanced chemiluminescence, and band densities were quantified by densitometry. The autoradiographs shown are representative of three independent experiments.

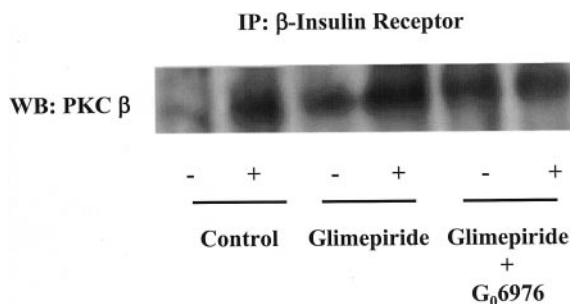


Fig. 10. Coprecipitation of the insulin receptor with PKC- β II isoform after treatment with PKC- β II inhibitor G06976. Hep-G2 cells cultured in the absence or presence of $20 \mu\text{mol/l}$ glimepiride for 72 were preincubated for 1 h with $2 \mu\text{M}$ PKC- β II inhibitor G06976, and further incubated in the presence or absence of 100 nM insulin for 30 min. The cells were lysed and equal amounts of proteins were immunoprecipitated with anti-insulin receptor antibody, separated by SDS-PAGE, transferred to nitrocellulose membrane, and Western immunoblotted with a specific PKC- β II antibody (Santa Cruz). Proteins were detected by using enhanced chemiluminescence, and band densities were quantified by densitometry. The autoradiographs shown are representative of three independent experiments.

glycogen in cells cultured with or without glimepiride in a dose-dependent manner. However, in the basal state, glucose incorporation into glycogen was 1.36-fold higher in glimepiride-treated cells compared with untreated cells (0.46 and 0.34 nmol/mg/min , $P < 0.03$, respectively). Treatment with glimepiride resulted also in a significant increase in insulin-stimulated incorporation of glucose into glycogen at any concentration tested ($n = 4$; $P < 0.001$ by two-way analysis of variance). Maximal insulin stimulation was 2.4-fold higher in glimepiride-treated cells than in control cells. The insulin sensitivity, estimated as the concentration of insulin required for half-maximal stimulation of glucose incorporation into glycogen (ED_{50}), was significantly higher in cells treated with glimepiride than in untreated cells ($\text{ED}_{50} = 4.2$ versus 12 nM ; $P < 0.001$, respectively). These results indicate that glimepiride increases both the responsiveness and the sensitivity to metabolic action of insulin. To address the question of whether PKC plays an important role in mediating the effects of glimepiride on glycogen synthesis, we analyzed insulin-stimulated glucose incorporation into glycogen after cell depletion of TPA-sensitive PKCs (PKC- β II and - ϵ) or inhibition of PKC- β II with G06976. As shown in Fig. 11, preincubation with TPA of glimepiride-treated cells almost completely reversed the effect of glimepiride to increase insulin-stimulated incorporation of glucose into glycogen at any concentration tested. Similarly, preincubation with G06976 inhibited the effect of glimepiride on insulin-stimulated glucose incorporation into glycogen (Fig. 11).

Discussion

Previous studies on the extrapancreatic actions of sulfonylureas in cultured cells have focused on the effects of these drugs on glucose transport and metabolism (Rogers et al., 1987; Muller and Wied, 1993; Tsiani et al., 1995). It has been reported that sulfonylureas directly stimulate glucose metabolism and potentiate insulin actions by enhancing GLUT-1 and GLUT-4 expression, translocation, and activation (Mul-

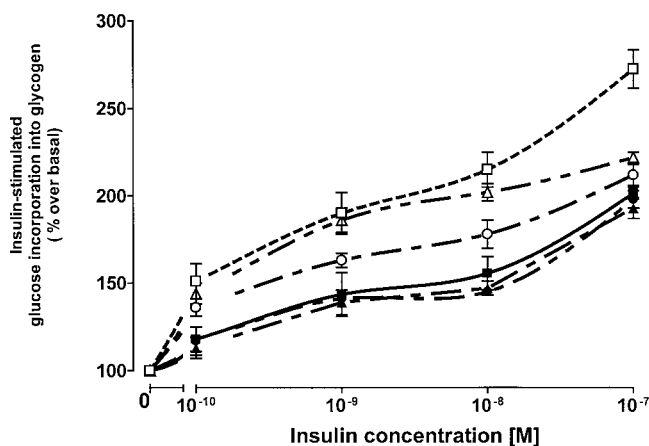


Fig. 11. Insulin-stimulated glucose incorporation into glycogen. Hep-G2 cells cultured in the absence (closed symbols) or presence (open symbols) of $20 \mu\text{mol/l}$ glimepiride for 72 were preincubated for 24 h with $1 \mu\text{M}$ TPA (\bullet , \circ) or for 1 h with $2 \mu\text{M}$ PKC- β II inhibitor G06976 (\blacktriangle , \triangle), and further incubated with DMEM/1% BSA containing glucose (2.5 mM final concentration) for 3 h at 37°C . Insulin at the indicated concentrations and [^{14}C]Glucose ($4 \mu\text{Ci}$) were then added to each well for 2 h. After cell solubilization, the radioactivity incorporated into glycogen was precipitated in ethanol and counted. Results are presented as the percentage over basal and are mean \pm S.D. of four experiments carried out in triplicate.

ler and Wied, 1993; Tsiani et al., 1995). Although most studies suggest that sulfonylureas exert these effects without affecting insulin binding (Bak et al., 1989) and insulin receptor tyrosine kinase activity (Jacobs et al., 1987), it is not known whether these drugs affect other postbinding events. In an attempt to clarify this issue, we investigated the effects of glimepiride on insulin-induced receptor internalization, receptor recycling, and insulin degradation in Hep-G2 human hepatoma cell line. This cell line has been chosen as an *in vitro* model, because liver is a major site of insulin degradation *in vivo*, and sulfonylureas are extensively metabolized in the liver with the metabolites and the parent drug being eliminated mainly in the urine. In addition, Hep-G2 human hepatoma cells are well differentiated, possess a large number of insulin receptors, maintain intracellular function and cell integrity after exposure to acid treatment, and have been widely used for studies on insulin action (Podskalny et al., 1985; McClain and Olefsky, 1988). We found that both recycling of the insulin receptor to the plasma membrane and release of intracellular processed insulin were increased in cells treated with glimepiride compared with control cells. This effect was dose- and time-dependent, requiring several hours of exposure to the drug. The effect of glimepiride was not associated with changes in insulin binding and insulin-receptor internalization, which is in accordance with results of previous studies (Jacobs et al., 1987; Bak et al., 1989). Glimepiride did not affect the ability of insulin to stimulate tyrosine phosphorylation of its receptor or activation of IRS-1 or IRS-2. Furthermore, glimepiride did not alter expression of the two insulin receptor isoforms, thus arguing against an explanation that increased expression of the Ex11⁺ insulin receptor isoform, which is known to possess slower rates of internalization and recycling (Yamaguchi et al., 1991), may account for the present results. Because the dissociation of insulin from its receptor is an essential step to allow both recycling of the receptor to the cell surface and release of processed insulin from the cell interior, an increased dissociation of insulin from the receptor within the endosome is one possible explanation for the results observed in cells treated with glimepiride. The present data obtained using a previously validated PEG-based assay (Levy and Olefsky, 1988) are consistent with this hypothesis. Therefore, in cells treated with glimepiride, an increased dissociation of insulin from its receptor results in both enhanced degradation of the internalized insulin and increased release of degradation products. As a consequence, a higher proportion of the internalized receptor is recycled back to the plasma membrane thus preventing receptor down-regulation during chronic insulin stimulation. To our knowledge, these findings provide the first direct evidence in cultured cells supporting a role for sulfonylureas in the intracellular processing of the insulin-receptor complex. Obviously, we cannot rule out that other explanations might account for the present results. For example, it has been reported that in the rat hepatoma cell line Fao, dissociation and degradation of internalized insulin occur in the endosomes, where insulin degradation facilitates insulin dissociation by reducing the endosomal concentration of intact insulin (Backer et al., 1990). Thus, glimepiride may mainly increase intracellular insulin degradation in the endosomal compartment that, in turn, increases the extent of ligand dissociation from the receptor. Alternatively, although we could not detect any changes in the relative abundance of

the two insulin receptor isoforms, we cannot exclude the idea that subtle differences in insulin-receptor binding affinity may affect the sensitivity of the internalized insulin-receptor complex to modifications in pH, thus altering the rate at which insulin dissociates from its receptor.

The biochemical signals that determine intracellular routing of the insulin receptor are not completely defined. Recently, it has been reported that interaction of PKCs with the insulin receptor may have an important role in regulating intracellular sorting of the internalized insulin-receptor complexes (Formisano et al., 1998). We found that glimepiride increased the insulin-induced association of PKC- β II and - ϵ with the insulin receptor without affecting total cellular content. Cellular depletion of PKCs by treatment with TPA reduced the amounts of PKC- β II and - ϵ coprecipitating with the insulin receptor upon insulin stimulation. Moreover, treatment with TPA almost completely reversed the effect of glimepiride to increase insulin receptor recycling, release of intracellular insulin, and degradation of intracellular insulin. Treatment of cells with PKC- β II inhibitor G06976 also inhibited insulin-induced coprecipitation of PKC- β II with the insulin receptor, suggesting that the insulin-induced activation of this PKC isoform is necessary to allow its subsequent association with the receptor. G06976 treatment of the cells also reversed the effect of glimepiride on insulin-insulin receptor processing. Based on the present and previous results (Formisano et al., 1998), it is reasonable to speculate that sulfonylureas may regulate the intracellular sorting of the insulin-receptor complexes toward the degradative compartment by a mechanism that involves PKCs, thus unveiling an important biochemical and functional link between PKC system and the insulin receptor.

A question that arises from the present results is whether the effects of sulfonylurea on the processing of the insulin-receptor complex are relevant to *in vivo* insulin action. Previous *in vivo* and *in vitro* investigations have provided substantial evidence for a relationship between insulin action and intracellular processing of insulin and its receptor (Ferrannini et al., 1982; Flier et al., 1982; Peavy et al., 1984; Veda et al., 1985; Jochen and Berhanu, 1987; Miller, 1988). Furthermore, it has been suggested that abnormalities in insulin receptor recycling and intracellular processing of the insulin-receptor complex might contribute to impair insulin action in patients with type 2 diabetes mellitus (Jochen et al., 1986; Grunberger et al., 1989; Trischitta et al., 1989; Benzi et al., 1990, 1997; Sesti et al., 1996). We found that treatment with glimepiride causes an increase in both insulin sensitivity and responsiveness for glucose incorporation into glycogen. Exposing cells to TPA or G06976 inhibitor reversed these effects. These findings are consistent with those of a preliminary study showing that the reduced intracellular degradation of insulin observed in isolated monocytes from patients with type 2 diabetes mellitus can be ameliorated by sulfonylurea treatment in parallel with improvement of glucose tolerance (Ciccarone et al., 1987). However, because the 2.4-fold increase in insulin-stimulated incorporation of glucose into glycogen was not associated with a proportionate increase in intracellular insulin processing, it is likely that glimepiride also affects additional cellular processes that influence glucose metabolism such as glucose transport (Muller and Wied, 1993).

Receptor-mediated insulin endocytosis is the principal mechanism for insulin clearance from the blood, and evi-

dence has been provided that this process is impaired in patients with type 2 diabetes (Jochen et al., 1986; Grunberger et al., 1989; Trischitta et al., 1989; Benzi et al., 1990, 1997). Thus, regulation by sulfonylureas of intracellular processing of insulin might have profound pathophysiological consequences by modulating the concentration of insulin in the peripheral circulation and avoiding the deleterious effects of hyperinsulinemia. Taken together, these data raise the possibility that sulfonylurea-induced changes in the intracellular processing of insulin and its receptor may have a role in improving insulin action in peripheral tissues.

In conclusion, increased routing of the internalized insulin toward the degradative compartment involving the interaction of the insulin receptor with PKC isoforms might represent a novel mechanism of action of sulfonylureas. Given the evidence for a relationship between the biological activity of insulin and the intracellular processing of insulin and its receptor, the present results may have a therapeutic impact on the treatment of insulin-resistant states.

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References

- Backer JM, Kahn CR and White MF (1990) The dissociation and degradation of internalized insulin occur in the endosomes of rat hepatoma cells. *J Biol Chem* **265**:14828–14835.
- Bak JF, Schmitz O, Sorensen NS and Pedersen O (1989) Postreceptor effects of sulfonylurea on skeletal muscle glycogen synthase activity in type II diabetic patients. *Diabetes* **38**:1343–1350.
- Benzi L, Trischitta V, Ciccarone AM, Cecchetti P, Brunetti A, Squatrito S, Marchetti P, Vigneri R and Navalesi R (1990) Improvement with metformin in insulin internalization and processing in monocytes from NIDDM patients. *Diabetes* **39**:844–849.
- Benzi L, Cecchetti P, Ciccarone AM, Nardone A, Merola E, Maggiorelli R, Campi F, Di Cianni G and Navalesi R (1997) Inhibition of endosomal acidification in normal cells mimics the derangements of cellular insulin and insulin-receptor metabolism observed in non-insulin-dependent diabetes mellitus. *Metabolism* **46**:1259–1265.
- Carpentier JL (1994) Insulin receptor internalization: Molecular mechanisms and pathophysiological implications. *Diabetologia* **37**(Suppl 2):S117–S124.
- Ciccarone AM, Benzi L, Cecchetti P, Trischitta V, Masoni A, Di Cianni G and Navalesi R (1987) Effects of sulfonylurea on intracellular processing of insulin in monocytes from Type II (non-insulin-dependent) diabetic patients. *Diabetologia* **30**:507A.
- Cooper DR, Vila MC, Watson JE, Nair G, Pollet RJ, Standaert M and Farese RV (1990) Sulfonylurea-stimulated glucose transport association with diacylglycerol-like activation of protein kinase C in BC3H1 myocytes. *Diabetes* **39**:1399–1407.
- Ducher L, Croquet F, Gil S, Davy J, Feger J and Brehier A (1995) Differential expression of five protein kinase C isoenzymes in Fao and Hep-G2 hepatoma cell lines compared with normal rat hepatocytes. *Biochem Biophys Res Commun* **217**:546–553.
- Ferrannini E, Muggeo M, Navalesi R and Pilo A (1982) Impaired insulin degradation in a patient with insulin resistance and acanthosis nigricans. *Am J Med* **73**:148–154.
- Flier JS, Minaker KL, Landsberg L, Young JB, Pallotta J and Rowe LW (1982) Impaired in vivo insulin clearance in patients with severe target-cell resistance to insulin. *Diabetes* **31**:132–135.
- Formisano P, Oriente F, Miele C, Caruso M, Auricchio R, Vigliotta G, Condorelli G and Beguinot F (1998) In NIH-3T3 fibroblasts, insulin receptor interaction with specific protein kinase C isoforms controls receptor intracellular routing. *J Biol Chem* **273**:13197–13202.
- Grunberger G, Geiger D, Carpentier JL, Robert A and Gorden P (1989) Receptor-mediated endocytosis of insulin: Inhibition of (¹²⁵I)iodoinsulin internalization in insulin resistant diabetic states of man. *Acta Endocrinol* **121**:581–586.
- Hirshman MF and Horton ES (1990) Glyburide increases insulin sensitivity, responsiveness in peripheral tissues of the rat as determined by the glucose clamp technique. *Endocrinology* **126**:2407–2412.
- Jacobs DB, Hayes GR and Lockwood DH (1987) Effect of chlorpropamide on glucose transport in rat adipocytes in the absence of changes in insulin binding and receptor-associated tyrosine kinase activity. *Metabolism* **36**:548–554.
- Jochen AL, Berhanu P and Olefsky JM (1986) Insulin internalization and degradation in adipocytes from normal and type II diabetic subjects. *J Clin Endocrinol Metab* **62**:268–272.
- Jochen AL and Berhanu P (1987) Insulin-stimulated glucose transport and insulin internalization share a common postbinding step in adipocytes. *Diabetes* **36**:542–545.
- Kolterman OG, Gray RS, Shapiro G, Scarlett J, Griffin J and Olefsky JM (1984) The acute and chronic effects of sulfonylurea therapy in type II diabetic subjects. *Diabetes* **33**:346–354.
- Lebovitz HE (1984) Cellular loci of sulfonylurea actions. *Diabetes Care* **7** (Suppl 1):67–71.
- Levy JR and Olefsky JM (1987) The trafficking and processing of insulin and insulin receptors in cultured rat hepatocytes. *Endocrinology* **121**:2075–2086.
- Levy JR and Olefsky JM (1988) Intracellular insulin-receptor dissociation and segregation in a rat fibroblast cell line transfected with a human insulin receptor gene. *J Biol Chem* **263**:6101–6108.
- Marshall S (1985a) Dual pathways for the intracellular processing of insulin. *J Biol Chem* **260**:13524–13531.
- Marshall S (1985b) Kinetics of insulin receptor internalization and recycling in adipocytes. *J Biol Chem* **260**:4136–4143.
- McClain DA and Olefsky JM (1988) Evidence for two independent pathways of insulin-receptor internalization in hepatocytes and hepatoma cells. *Diabetes* **37**:806–815.
- Miller DS (1988) Stimulation of RNA and protein synthesis by intracellular insulin. *Science (Wash DC)* **240**:506–509.
- Muller G and Wied S (1993) The sulfonylurea drug, glimepiride, stimulates glucose transport, glucose transporter translocation, and dephosphorylation in insulin-resistant rat adipocytes in vitro. *Diabetes* **42**:1852–1866.
- Peavy DE, Edmondson JW and Duckworth WC (1984) Selective effects of inhibitors of hormone processing on insulin action in isolated hepatocytes. *Endocrinology* **114**:753–760.
- Podskalny JM, Takeda S, Silverman RE, Tran D, Carpentier J-L, Orci L and Gorden P (1985) Insulin receptors and bioresponses in a human liver cell line (Hep G-2). *Eur J Biochem* **150**:401–407.
- Rogers BJ, Standaert ML and Pollet RJ (1987) Direct effects of sulfonylurea agents on glucose transport in the BC3H-1 myocyte. *Diabetes* **36**:1292–1296.
- Rother KI, Imai Y, Caruso M, Beguinot F, Formisano P and Accili D (1998) Evidence that IRS-2 is required for insulin action in hepatocytes. *J Biol Chem* **273**:17491–17497.
- Seedorf K, Shearman M and Ullrich A (1995) Rapid and long-term effects on protein kinase C on receptor tyrosine kinase phosphorylation and degradation. *J Biol Chem* **270**:18953–18960.
- Sesti G, D'Alfonso R, Vargas Punti MD, Tullio AN, Liu YY, Federici M, Borboni P, Marini MA, Lauro R, Fusco A (1996) Delayed intracellular dissociation of the insulin-receptor complex impairs receptor recycling and insulin processing in cultured EBV-transformed lymphocytes from insulin-resistant subjects. *Diabetologia* **39**:289–297.
- Sesti G, Marini MA, Montemurro A, Borboni P, Condorelli L, Haring HU, Ullrich A, Goldfine D, De Pirro R and Lauro R (1992) Evidence that two naturally occurring human insulin receptor α -subunit variants are immunologically distinct. *Diabetes* **41**:6–11.
- Sesti G, Tullio AN, D'Alfonso R, Napolitano ML, Marini MA, Borboni P, Longhi R, Albonici L, Fusco A, Aglianò AM, et al (1994a) Tissue-specific expression of two alternatively spliced isoforms of the human insulin receptor protein. *Acta Diabetol* **31**:59–65.
- Sesti G, Tullio AN, Marini MA, Manera E, Borboni P, Accili D, Longhi R, Fusco A, Lauro R and Montemurro A (1994b) Role of the exon 11 of the insulin receptor gene on insulin binding identified by anti-peptide antibodies. *Mol Cell Endocrinol* **101**:121–127.
- Simonson D, Ferrannini E, Stefano S, Smith D, Barret E, Carlson R and DeFronzo RA (1984) Mechanism of improvement in glucose metabolism after chronic glyburide therapy. *Diabetes* **32**:35–45.
- Trischitta V, Brunetti A, Chiavetta A, Benzi L, Papa V and Vigneri R (1989) Defects in insulin-receptor internalization and processing in monocytes of obese subjects and obese NIDDM patients. *Diabetes* **38**:1579–1584.
- Tsiani E, Ramlal T, Leiter LA, Klip A and Fantus IG (1995) Stimulation of glucose uptake and increased plasma membrane content of glucose transporters in L6 skeletal muscle cells by the sulfonylureas gliclazide and glyburide. *Endocrinology* **136**:2505–2512.
- Ullrich A, Bell JR, Chen EY, Herrera R, Petruzzelli LM, Dull TJ, Gray A, Coussens L, Liao YC, Tsubokawa M, et al (1985) Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. *Nature (Lond)* **313**:756–761.
- Veda M, Robinson R, Smith MM and Kono T (1985) Effects of monensin on insulin processing in adipocytes: Evidence that the internalized insulin-receptor complex has some biological activities. *J Biol Chem* **260**:3941–3946.
- Yamaguchi Y, Flier JS, Yokota A, Benecke H, Backer JM and Moller DE (1991) Functional properties of two naturally occurring isoforms of the human insulin receptor in Chinese hamster ovary cells. *Endocrinology* **129**:2058–2066.

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