Insulinlike Activity of New Antidiabetic Agent CP 68722 in 3T3-L1 Adipocytes

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We examined the in vitro effects of CP 68722, a novel antidiabetic agent, in 3T3-L1 adipocytes. CP 68722 stimulated 2-deoxyglucose uptake in the absence of insulin. At least 30 min of incubation were required for stimulation of uptake. This effect increased over 5 h and was sustained up to 72 h. The stimulation of 2-deoxyglucose uptake by CP 68722 could be inhibited ~60% by inhibition of protein synthesis with cycloheximide. Half-maximal and maximal responses to CP 68722 at 72 h of incubation were observed at 10 and 100 μM of drug, respectively, with a threefold stimulation of uptake at 100 μM approximating the maximal response of these cells to acute insulin stimulation. CP 68722 was able to overcome insulin resistance induced by dexamethasone in 3T3-L1 cells. The effect of drug, like that of insulin, was primarily to increase the V_{max} of 2-deoxyglucose uptake. The stimulation of uptake by CP 68722 or insulin could be prevented by incubating the cells at 10°C, a temperature that impedes translocation of glucose transporters to the plasma membrane. Therefore, it appears that CP 68722, like insulin, stimulates glucose uptake by a mechanism that involves translocation of intracellular glucose transporters to the plasma membrane and de novo protein synthesis. We compared the effect of CP 68722 with the sulfonylureas, the primary drugs used in the treatment of non-insulin-dependent diabetes mellitus (NIDDM). CP 68722 was a more potent and effective stimulator of 2-deoxyglucose uptake in 3T3-L1 cells than either first- or second-generation sulfonylureas. Our results suggest that CP 68722 could be an effective therapeutic agent for the treatment of NIDDM. *Diabetes* 39:1414–19, 1990

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Non-insulin-dependent diabetes mellitus (NIDDM) is characterized by three major metabolic defects: peripheral insulin resistance, enhanced hepatic glucose output, and abnormal pancreatic insulin secretion (1). At the molecular level, peripheral insulin resistance has been associated with defects in the glucose transport system. In fat cells taken from humans with NIDDM, there is a decrease in the maximal rate of insulin-stimulated glucose transport (2). This transport defect is associated with a specific decrease in the intracellular pool of glucose transporters, resulting in fewer transporter molecules available for translocation to the plasma membrane in response to insulin (3). Similar defects in glucose transport have been observed in animal models of insulin resistance associated with insulin deficiency and insulin excess (4–7).

CP 68722 [(±)-5-[(3,4-dihydro-2-phenylmethyl-2H-1-benzopyran-6-yl)methyl]-thiazolidine-2,4-dione] is an orally active agent for the treatment of NIDDM. Like other thiazolidinediones (e.g., ciglitazone), it acts by enhancing the action of insulin without stimulating insulin secretion from the pancreas (8–10). In the *ob/ob* mouse, CP 68722 normalizes hyperglycemia and significantly reduces hyperinsulinemia in this model of NIDDM (11). The reduction in plasma levels of glucose and insulin in response to this drug suggests that the sensitivity of the peripheral tissues to insulin is increased. CP 68722 has similar activity in the hyperinsulinemic fatty Zucker rat model of insulin resistance. The impaired glucose tolerance found in this animal model is improved by CP 68722, suggesting that glucose disposal was enhanced (unpublished observations).

In this study, we examined the direct effects of CP 68722 on glucose transport in vitro using the 3T3-L1 cell line established by Green and Kehinde (12). This cell line is a well-defined system in which to study insulin action and factors regulating glucose transport. As differentiated adipocytes, these cells express increased insulin receptors and insulin-stimulated glucose transport and oxidation (13,14). In ad-

dition, the cells can be rendered insulin resistant with dexamethasone (15,16). We examined the effects of CP 68722 on 2-deoxyglucose (2-DG) uptake into 3T3-L1 cells in both their native and insulin-resistant states and compared the effects of this agent with first- and second-generation sulfonylureas, the most common pharmacological agents used for the treatment of NIDDM (17).

RESEARCH DESIGN AND METHODS

3T3-L1 cells (CL 173, American Type Culture Collection, Rockville, MD) were grown in Dulbecco's modified Eagle's medium (25 mM glucose) containing 10% fetal bovine serum and differentiated essentially as described by van Putten et al. (16). Briefly, 2 days postconfluence, the cells were treated with 0.5 mM 1-methyl-3-isobutylxanthine, 250 μM dexamethasone, and 10 μg/ml insulin for 2 days followed by insulin alone for 2 days. Cells were incubated in the absence of insulin for 5–7 days before initiation of experiments. Cells were treated by adding drug dissolved in dimethylsulfoxide directly to the culture media. Fresh drug in media was added daily. The concentration of dimethylsulfoxide in media did not exceed 0.2%, which had no effect on the rate of 2-DG uptake into the cells.

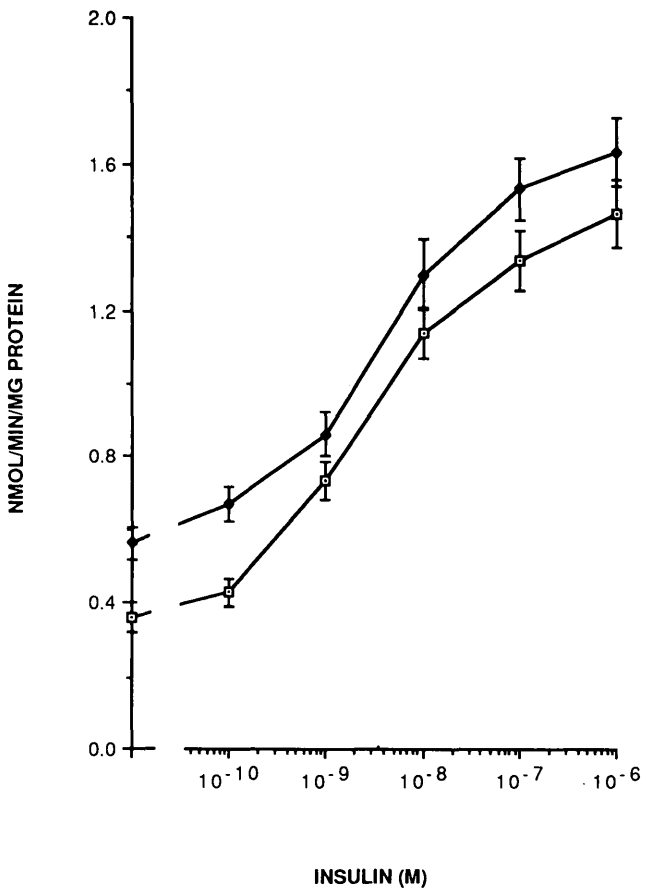

FIG. 1. Stimulation of 2-deoxyglucose uptake by insulin and 10 μM CP 68722. Cells were treated as described in RESEARCH DESIGN AND METHODS and incubated with insulin for 30 min before addition of 0.2 mM [1-^{14}C]-2-deoxyglucose. Uptake was terminated after 10 min. Treatment with 10 μM CP 68722 was 72 h. Uptake in presence of 50 μM cytochalasin B was 0.05 and 0.08 nmol · min^{-1} · mg^{-1} protein in absence and presence of CP 68722, respectively. Results represent duplicate experiments with n = 6. □, Control; ●, CP 68722.

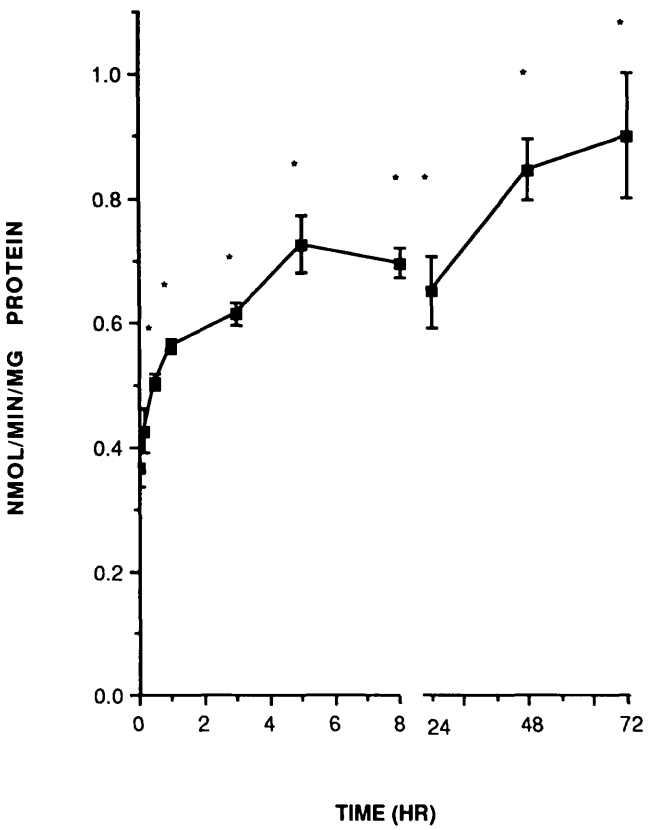

FIG. 2. Time course of CP 68722 action. Cells were treated with 30 μM CP 68722 for indicated periods. Deoxyglucose uptake was assayed as described in RESEARCH DESIGN AND METHODS and Fig. 1. Results represent duplicate experiments with n = 6. *$P < 0.05$.

2-DG uptake was measured in cells grown on 35-mm dishes (6-well cluster plates). The cell monolayer was washed three times with Dulbecco's phosphate-buffered saline (DPBS), and the cells were incubated for 30 min at 37°C in Krebs-Ringer HEPES buffer (or DPBS in Table 1) plus 2% bovine serum albumin, pH 7.4. Uptake was initiated by the addition of 0.2 mM [1-^{14}C]-2-DG (1 μCi/ml). After 10 min at 37°C, the medium was aspirated, the cells were washed three times with ice-cold DPBS and dissolved in 0.5% sodium dodecylsulfate, and an aliquot was taken for liquid-scintillation counting. DG uptake was linear for at least 45 min under these conditions.

RESULTS

Stimulation of 2-DG uptake by CP 68722. In various models of insulin resistance, at least part of the postreceptor defect can be attributed to aberrant glucose transport (2–7,18–20). We therefore examined the effect of CP 68722 on the glucose transport properties of 3T3-L1 adipocytes. Uptake of 2-DG into these cells was insulin sensitive; half-maximal stimulation was observed at ~3 nM insulin, and uptake was stimulated 4-fold by 1 μM hormone after 30 min of incubation (Fig. 1). CP 68722 also stimulated 2-DG uptake. As shown in Fig. 1, 10 μM CP 68722 stimulated uptake 1.8-fold in the absence of insulin, and the effect of drug was additive with that of insulin. Additivity of drug and insulin was also observed at higher concentrations of drug (21). Uptake in the presence

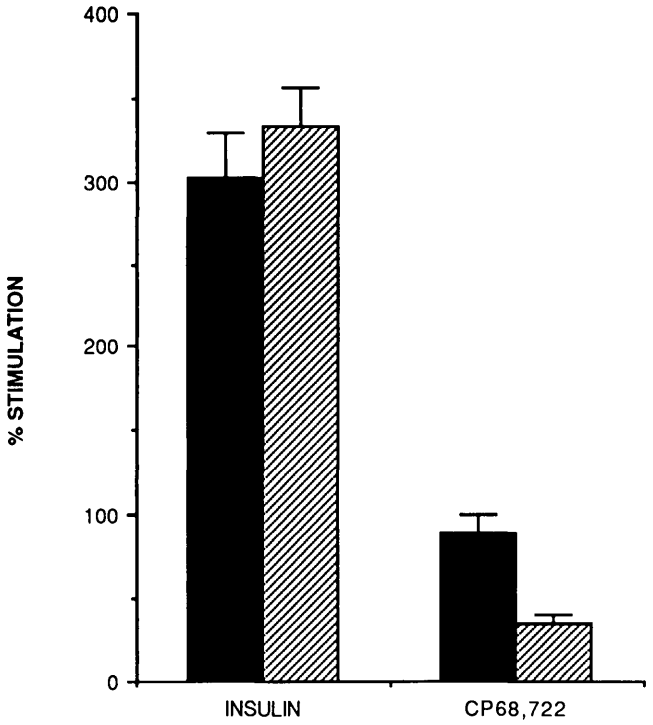

FIG. 3. Inhibition of CP 68722 action by cycloheximide. Cells were treated with 10 μg/ml cycloheximide for 3 h followed by 100 μM CP 68722 plus cycloheximide for additional 3 h In experiments with insulin, treatment with cycloheximide was for 5.5 h followed by 1 μM insulin plus cycloheximide for 30 min. Deoxyglucose uptake was assayed as described in RESEARCH DESIGN AND METHODS and Fig. 1. Results represent duplicate experiments with *n* = 6. *Solid bars*, control; *hatched bars*, cycloheximide.

and absence of drug was inhibited ~85% by cytochalasin B, indicating carrier-mediated glucose uptake.

The effect of CP 68722 developed slowly with time. The stimulatory effect of 30 μM CP 68722 increased during the first 5 h of incubation in the culture medium (Fig. 2). The earliest time at which a statistically significant drug effect was observed was 30 min. The stimulation at 5 h was twofold relative to control (t_0); this rate of uptake was sustained between 5 and 24 h of incubation. Between 24 and 72 h, there was a further stimulation of uptake. The slow onset of drug action suggested the involvement of protein synthesis, which was examined with cycloheximide. Cells were treated with 10 μg/ml cycloheximide for 3 h followed by 3 h with 100 μM CP 68722 plus cycloheximide. These incubation times were chosen to limit the cytotoxic effects of cycloheximide and yet allow sufficient time for stimulation of 2-DG uptake by CP 68722. Cycloheximide inhibited the incorporation of [^{3}H]methionine into protein by 85%, which was unaffected by insulin or CP 68722 (data not shown). Cycloheximide had no effect on the stimulation of 2-DG uptake by insulin (Fig. 3). After correcting for the 30% decrease in basal rate of uptake in the cycloheximide-treated cells, insulin stimulated 2-DG uptake fourfold in the presence and absence of cycloheximide. In contrast, inhibition of protein synthesis diminished the effect of CP 68722 by 60%. In the absence of cycloheximide, CP 68722 stimulated uptake by 87%, whereas in the presence of the inhibitor, stimulation was only 35%.

Concentration dependence of CP 68722 action. The stimulation of 2-DG uptake by CP 68722 was dependent on the concentration of drug in the incubation medium (Fig. 4). Uptake was stimulated 1.6-, 2.3-, and 3.1-fold by 10, 30, and 100 μM CP 68722, respectively, after 72 h of incubation with the cells. Thus, the effect of 100 μM CP 68722 was comparable to the effect of 1 μM insulin (Fig. 1).

Insulin-resistant 3T3-L1 cells. As reported by others (15,16), 100 nM dexamethasone for 48 h induced insulin resistance in 3T3-L1 cells (Fig. 5). Both basal and insulin-stimulated rates of DG uptake were reduced; the stimulation of 2-DG uptake by 1 μM insulin was decreased by 30%. CP 68722 at 10 μM partially prevented the effect of dexamethasone. Cells were treated with CP 68722 for 24 h and then with CP 68722 plus 100 nM dexamethasone for an additional 48 h. The basal rate of uptake was completely restored to its control value, and the responsiveness to insulin was improved by ~50% (Fig. 5).

Kinetic characterization and temperature dependence. The stimulation of DG uptake by CP 68722, as by insulin, was due to an increase in the V_{max} for transport. Figure 6 illustrates an Eadie-Hofstee plot of 2-DG uptake. Bearing in mind the limitations of measuring kinetic parameters for 2-DG uptake (20), we estimate from the *y*-intercept of the plot that the V_{max} is increased after 72 h of treatment with 10 μM CP 68722. In comparison, 1 μM insulin for 30 min increased the V_{max} to a greater extent than did CP 68722. The stimulation of DG uptake by CP 68722 or insulin could be prevented by treating the cells at 10°C, which inhibits the translocation of

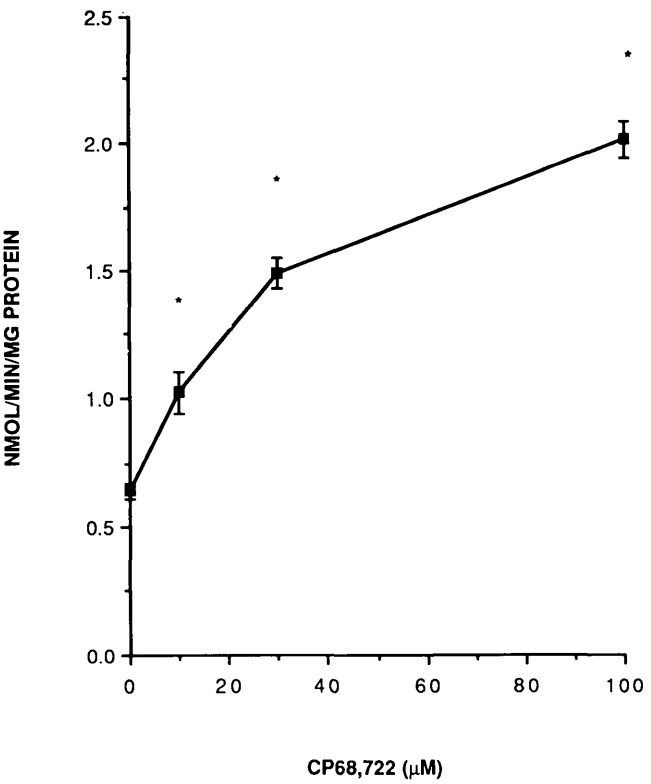

FIG. 4. Stimulation of 2-deoxyglucose uptake by CP 68722. Cells were incubated with CP 68722 for 72 h. Fresh drug was added every 24 h. Deoxyglucose uptake was assayed as described in RESEARCH DESIGN AND METHODS and Fig. 1. Results represent duplicate experiments with *n* = 6. *$P < 0.05$.

glucose transporters to the plasma membrane (22). At 37°C, 30 μM CP 68722 for 5 h stimulated cytochalasin B–sensitive 2-DG uptake 2.2-fold, whereas the same treatment at 10°C resulted in no stimulation; the control and drug-treated rates were identical (Table 1). The stimulation of 2-DG uptake by insulin was decreased by 90% when the temperature was lowered from 37 to 10°C.

Comparison with sulfonylureas. We compared the stimulation of DG uptake by CP 68722 and several sulfonylureas. CP 68722 was more potent and effective than either chlorpropamide, tolbutamide, or glipizide (Fig. 7). As expected, glipizide, a second-generation sulfonylurea, was more potent than either of the first-generation sulfonylureas. CP 68722 was at least one order of magnitude more potent than glipizide. In addition, the maximal rate of uptake observed after 72 h of treatment with CP 68722 was much greater than that observed after treatment with chlorpropamide, tolbutamide, or glipizide. Uptake was stimulated ~4-fold by 100 μM CP 68722 but only 2- to 2.5-fold by 1 mM sulfonylurea. Thus, CP 68722 is clearly more potent and effective than the sulfonylureas, although we cannot determine whether these differences are of a qualitative or quantitative nature.

DISCUSSION

Our results demonstrate that in 3T3-L1 cells, CP 68722 is a potent insulinomimetic agent at concentrations similar to serum concentrations that lower blood glucose in *ob/ob* mice (11; unpublished observations). Stimulation of uptake by CP 68722, like insulin, requires translocation of glucose carriers to the plasma membrane as evidenced by the lack of drug effect at low temperature and the increase in the V_{max} for uptake. It does not appear that CP 68722 activates preexisting glucose transporters in the plasma membrane, another feature that the drug shares with insulin. The effect of drug requires at least 30 min and increases over 5 h. These results suggest that translocation of glucose transporters from an intracellular pool to the plasma membrane is not the sole mechanism of drug action, because unlike insulin, CP 68722

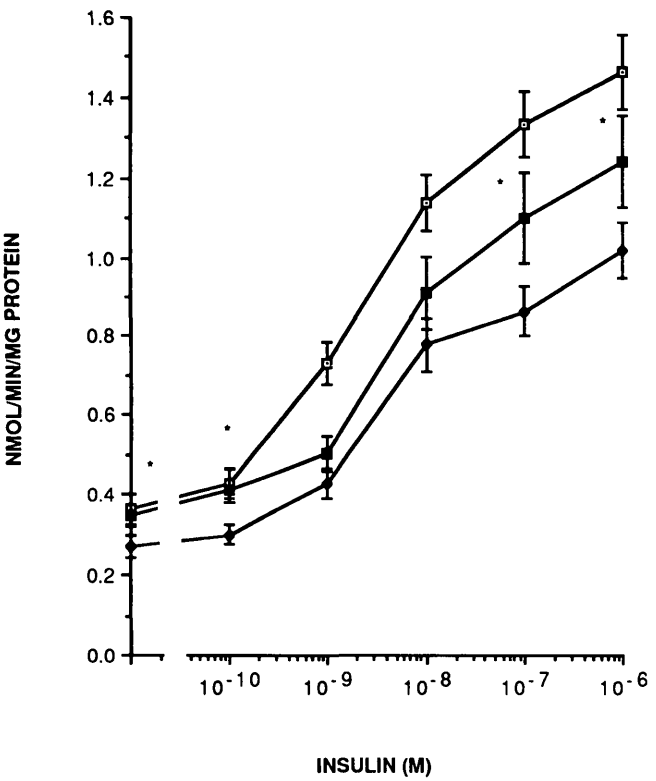

FIG. 5. Prevention of dexamethasone-induced insulin resistance by CP 68722. Cells were treated with 10 μM CP 68722 for 24 h and then with CP 68722 plus 100 nM dexamethasone for additional 48 h. Deoxyglucose uptake was assayed as described in RESEARCH DESIGN AND METHODS and Fig. 1. Results represent duplicate experiments with n = 6. □, Control; ●, dexamethasone; ■, dexamethasone plus CP 68722. *$P < 0.05$, dexamethasone vs. dexamethasone plus CP 68722.

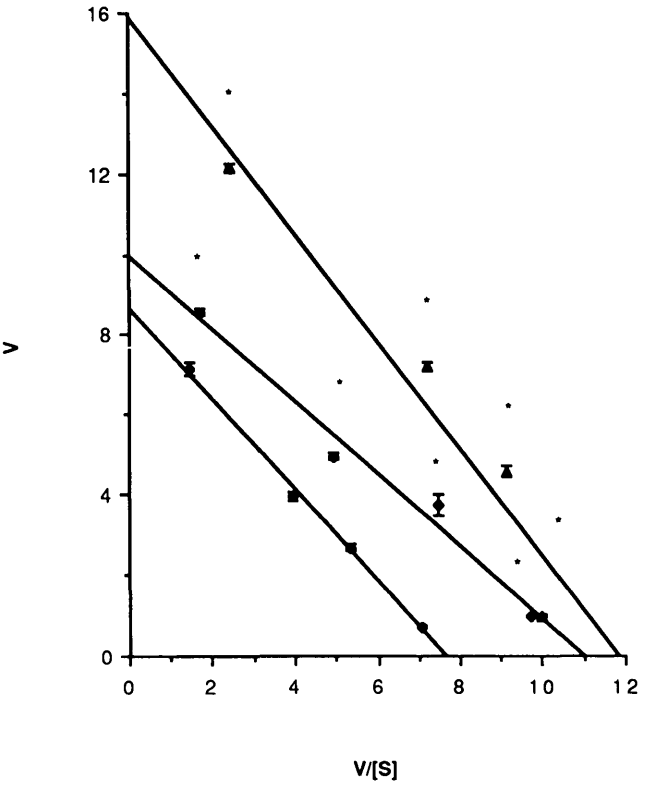

FIG. 6. Effect of CP 68722 on kinetics of 2-deoxyglucose uptake. Cells were incubated with CP 68722 for 72 h. Fresh drug was added every 24 h. Deoxyglucose uptake was assayed with 0.1, 0.5, 1, and 5 mM 2-deoxyglucose as described in RESEARCH DESIGN AND METHODS and Fig. 1. Results are from representative experiment with triplicate determinations. ●, Control; ♦, CP 68722; ▲, insulin. *$P < 0.05$.

TABLE 1
Low-temperature inhibition of CP 68722 and insulin action

	37°C (pmol · min^{-1} · mg^{-1} protein)	10°C (pmol · min^{-1} · mg^{-1} protein)
Control	134 ± 0.11	166 ± 0.11
30 μM CP 68722	284 ± 0.43*	171 ± 0.37†
0.1 μM insulin	1809 ± 2.20*	246 ± 0.32*

Values are means ± SE of 3 independent experiments with triplicate measures. Cells were maintained at 10 or 37°C for 5 h, during which they were treated with CP 68722 for the entire period or with insulin for the final 30 min. Uptake of 0.1 mM 2-deoxyglucose was measured for 10 min at 10 or 37°C. Rates of uptake were corrected for diffusion by subtraction of uptake in the presence of 50 μM cytochalasin B. Incubation buffer was switched from HEPES to phosphate, which resulted in a decrease in the basal rate of 2-deoxyglucose uptake, and therefore, a greater-fold stimulation by insulin.
*$P < 0.005$, †$P > 0.1$, vs. control.

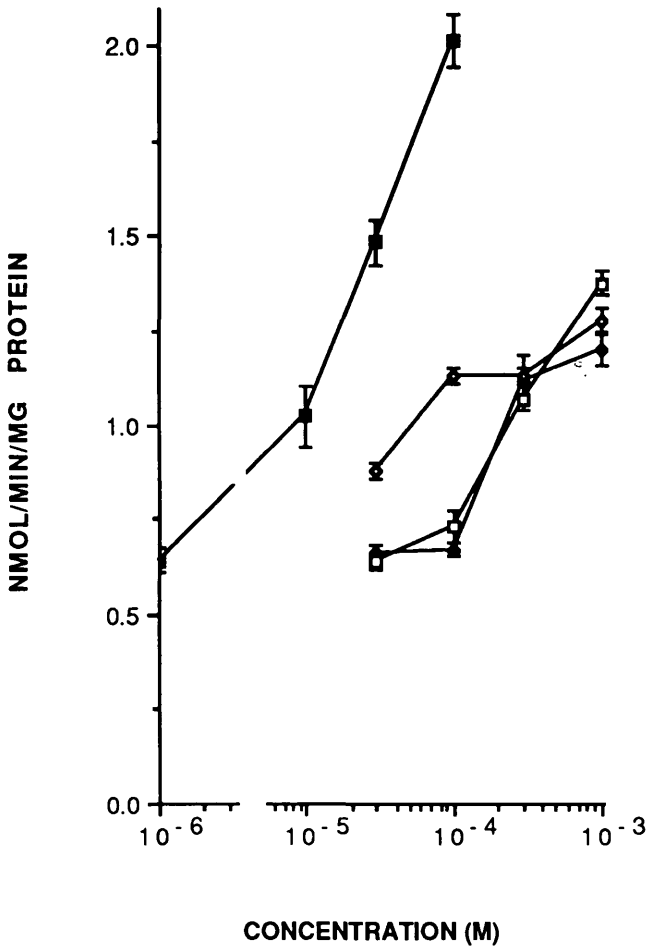

FIG. 7. Comparison of CP 68722 with sulfonylureas. Cells were treated with indicated concentrations of drug for 72 h. Fresh drug was added every 24 h. Deoxyglucose uptake was assayed as described in RESEARCH DESIGN AND METHODS and Fig. 1. Results represent duplicate experiments with n = 6. ■, CP 68722; ◇, glipizide; ♦, tolbutamide; □, chlorpropamide.

has no significant effect on 2-DG uptake after a 30-min incubation.

The slow time course for CP 68722 action suggests that de novo synthesis of glucose carriers might be induced. This hypothesis is supported by the observation that pretreatment of the cells with the protein synthesis inhibitor cycloheximide decreases amino acid incorporation into protein by 85% and inhibits the stimulation of 2-DG uptake by CP 68722. We confirmed these observations by directly measuring the cellular content of glucose transporters in response to CP 68722 (21). However, the suppression of drug action by cycloheximide is not complete. It is unclear whether this is due to protein synthesis–independent actions of drug or to incomplete inhibition of protein synthesis by 10 μg/ml cycloheximide. Thus, despite the similarities between the effects of CP 68722 and insulin, there are clear differences in the mechanisms by which the two agents stimulate glucose transport in 3T3-L1 cells.

Because we demonstrated that CP 68722 alleviates insulin resistance in *ob/ob* mice and fatty Zucker rats, two animal models of NIDDM (11; unpublished observations), we determined whether the drug could overcome insulin resistance in cells treated with dexamethasone. As observed by others with 3T3-L1 cells (15,16) and rat adipocytes (18,19), the insulin responsiveness of glucose transport is reduced by dexamethasone treatment, i.e., the cells become insulin resistant. CP 68722 is able to overcome this resistance, supporting our in vivo observations. We also employed a different experimental paradigm in which we treated cells with dexamethasone for various periods and then attempted to reverse the resistance with CP 68722. We observed that the greater the degree of insulin resistance, the higher the concentration of drug required to reverse it (unpublished observations), suggesting that the drug acts at the level of insulin resistance rather than simply exerting additive effects with insulin. The effect of dexamethasone on 3T3-L1 cells is to alter the maximal response to insulin, rather than the sensitivity to insulin, suggesting the induction of a postreceptor defect (23). Thus, it appears that CP 68722 exerts its effects in 3T3-L1 cells at a postreceptor site.

Sulfonylureas are the most commonly prescribed drugs for the treatment of NIDDM. We compared the effect of CP 68722 with the first- and second-generation sulfonylureas on glucose transport in 3T3-L1 cells. CP 68722 is clearly superior to the sulfonylureas in this experimental system. In the absence of insulin, tolbutamide, chlorpropamide, and glipizide stimulate DG uptake 2- to 2.5-fold, consistent with the observations of Zuber et al. (24). As has been demonstrated for sulfonylureas (25), stimulation of glucose uptake by CP 68722 appears to involve translocation of glucose carriers from an intracellular site to the plasma membrane. However, the effects of CP 68722 on glucose uptake are significantly greater and are observed at lower concentrations of drug than with the sulfonylureas. In addition, the sulfonylureas require the addition of insulin to exert full action. In the absence of insulin, sulfonylureas induce the expression of the glucose-transporter gene and increase the amount of glucose-transporter protein, but these new carriers remain in an intracellular site. Translocation to the plasma membrane requires the addition of insulin (26). Our results suggest that CP 68722 is able to exert both effects independent of insulin.

Two other drugs of the same chemical class as CP 68722 have been reported to have glucose-lowering properties in animal models of insulin resistance. Ciglitazone and CS-045 reduce plasma glucose and insulin in *KK-A^y* mice and improve glucose tolerance in fatty Zucker rats (8,9). In addition, adipocytes from CS-045–treated animals show increased rates of 2-DG uptake. This effect appears to be of the same magnitude as that observed with CP 68722, except that the stimulation of glucose uptake by CP 68722 is observed after in vitro treatment with drug in the absence of insulin. Ciglitazone has no insulinomimetic effects after 24 h of incubation with cultured rat adipocytes (10). Thus, ours is the first in vitro demonstration that this class of agents has an insulinomimetic action. Our results illustrate the utility of the 3T3-L1 cell line in its native and insulin-resistant state as a model system for studying the mechanism of action of hypoglycemic agents and suggest that CP 68722, and the thiazolidinediones in general, could be effective therapeutic agents for the treatment of NIDDM with more potent effects on the glucose transport apparatus than the sulfonylureas.

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