Modulation of Glycogen Phosphorylase Activity and Fructose 2,6-Bisphosphate Levels by Glibenclamide and Meglitinide in Isolated Rat Hepatocytes: A Comparative Study

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The influence of glibenclamide and meglitinide, or 4-[2-(5-chloro-2-methoxybenzamide)ethyl]-benzoic acid, a compound similar to the nonsulfonylurea moiety of glibenclamide, on glycogen phosphorylase *a* activity, fructose 2,6-bisphosphate (F-2,6-P_2) level, and cytoplasmic free-Ca^{2+} concentration has been studied in isolated rat hepatocytes. Both glibenclamide and meglitinide caused a transient and dose-dependent activation of glycogen phosphorylase, with half-maximal effects corresponding to 3.7 ± 1.6 and 9.6 ± 3.3 μmol/L, respectively. This enzyme activation occurred without significant changes in hepatocyte cyclic adenosine monophosphate (cAMP) levels and was accompanied by an increase in cytoplasmic concentration of free Ca^{2+}. Parallel to these effects, glibenclamide increased the cellular content of F-2,6-P_2, with this effect being associated with a reduction in the rate of glucose formation from a mixture of [^{14}C]lactate/pyruvate. Under similar conditions, meglitinide caused a significant reduction of F-2,6-P_2 levels and accelerated the gluconeogenic flux. The mechanism by which meglitinide decreases hepatocyte F-2,6-P_2 levels seems to be mediated by stimulation of fructose-2,6-bisphosphatase. This comparative study may help to elucidate which among the hepatic effects of glibenclamide are exerted specifically by the sulfonylurea moiety.

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IT IS WELL ESTABLISHED that the major hypoglycemic action of sulfonylureas—a family of therapeutic agents extensively used in the treatment of non–insulin-dependent diabetes mellitus[1]—is mediated by the stimulation of insulin secretion. However, different reports have demonstrated that these hypoglycemic agents may increase the concentration of fructose 2,6-bisphosphate (F-2,6-P_2) in perfused rat livers[2,3] and in both isolated[4-7] and cultured[8] rat hepatocytes. In agreement with the physiologic role that F-2,6-P_2 plays in the regulation of hepatic glucose metabolism,[9] it has been demonstrated that the increase in hepatic F-2,6-P_2 caused by sulfonylureas is closely related to the acceleration of glycolysis and the reduction of gluconeogenesis.[4,6-8]

In relation to the mechanism by which sulfonylureas increase hepatic F-2,6-P_2 levels, it has been reported that tolbutamide—in the millimolar range—activates 6-phosphofructo-2-kinase and inactivates fructose-2,6-bisphosphatase in isolated rat hepatocytes.[5,7] Furthermore, it has been demonstrated that sulfonylureas may cause activation of glycogen phosphorylase in hepatocytes isolated from fed rats, with a consequent increase in the cellular pool of hexose-6-phosphate.[10] In this way, the paradoxic glycogenolytic effect of sulfonylureas could collaborate to increase hepatocyte F-2,6-P_2 levels.[10] It must be mentioned that the activation of glycogen phosphorylase by sulfonylureas seems to be a Ca^{2+}-dependent process associated with an increase in the cytosolic free-Ca^{2+} concentration ($[Ca^{2+}]_i$).[11]

On the other hand, it is well established that meglitinide, or 4-[2-(5-chloro-2-methoxybenzamide)ethyl]-benzoic acid, which is similar to the nonsulfonylurea moiety of glibenclamide (Fig 1), also presents hypoglycemic properties.[12] This hypoglycemic action has been demonstrated to be due to stimulation of insulin release by a mechanism similar to that elicited by sulfonylureas.[13-16]

To elucidate which among the hepatic actions of glibenclamide are exerted specifically by the sulfonylurea moiety, we have investigated the effect of glibenclamide and that of meglitinide on glycogen phosphorylase *a* activity, F-2,6-P_2 level, and $[Ca^{2+}]_i$ in isolated rat hepatocytes. Our results have demonstrated that both glibenclamide and meglitinide caused an increase in $[Ca^{2+}]_i$ and consequently provoked a Ca^{2+}-dependent activation of glycogen phosphorylase. These findings indicate that the modulation of Ca^{2+} fluxes can be ascribed to the nonsulfonylurea moiety of glibenclamide, as previously demonstrated in studies performed in pancreatic β cells.[13-16] With respect to the influence on F-2,6-P_2 levels, glibenclamide—as expected—increased the concentration of this regulatory metabolite. In contrast, meglitinide caused a significant reduction in hepatocyte F-2,6-P_2 levels, as a result of the stimulation of fructose-2,6-bisphosphatase activity.

MATERIALS AND METHODS

Reagents

Collagenase A was purchased from Boehringer Mannheim (Mannheim, Germany). Glibenclamide (lot F/2975) and meglitinide were kindly supplied by Boehringer Mannheim and Hoechst Ibérica (Barcelona, Spain), respectively. Fura-2 acetoxymethyl ester and pluronic F-127 were obtained from Molecular Probes (Eugene, OR). The remaining reagents, all of analytic grade, were from Boehringer, Sigma (St Louis, MO), or Merck (Darmstadt, Germany). On the day of the experiment, fresh solutions of both glibenclamide and meglitinide in 10 mmol/L NaOH were prepared.

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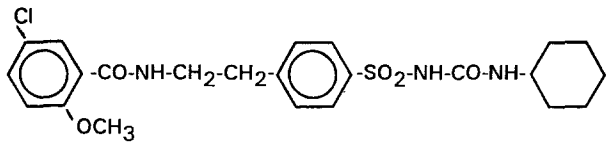
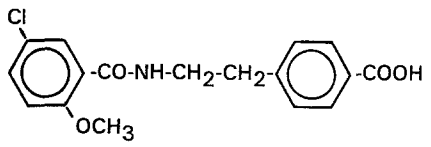


GLIBENCLAMIDE



MEGLITINIDE

Fig 1. Structural formulae of glibenclamide and meglitinide.

Animals

Fed male Wistar rats (200 to 300 g) from our inbred colony were used. The animals were maintained on a standard chow (RMN Labsure; Biosure, Barcelona, Spain) and water ad libitum.

Hepatocyte Isolation and Cell Incubations

Hepatocytes were isolated by perfusion of the liver with collagenase.[17] Isolated cells were suspended in Krebs-Henseleit medium, and viability was evaluated by the Trypan blue test; usually, 90% to 95% of the cells excluded the stain. Samples of cell suspensions (1 to 2 mL) containing 40 to 80 mg cells were incubated in the presence of 10 mmol/L glucose in stoppered 20-mL vials at 37°C with agitation (120 strokes per minute). The gas phase was 95% O_2/5% CO_2. After a 30-minute preincubation of cell suspensions, 20-μL aliquots of the corresponding solutions of glibenclamide, meglitinide, or vehicle (10 mmol/L NaOH) were added (zero time), and the incubation continued for the indicated times.

Metabolite Assays

Hepatocyte F-2,6-P_2 level was measured by the ability of this metabolite to activate potato tuber PPi:fructose-6-phosphate-1-phosphotransferase[18] according to the method previously described.[4] For determination of other metabolites, aliquots of cell incubations were taken at the selected times and immediately deproteinized in 5% $HClO_4$. After neutralization of the extracts,[4] fructose 6-phosphate, adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) levels were determined by enzymatic methods.[19-21] Gluconeogenesis was estimated by the rate of [U-^{14}C]lactate conversion to [^{14}C]glucose, as previously indicated[4]; the gluconeogenic precursor was a mixture of [U-^{14}C]lactate/pyruvate (4/0.4 mmol/L, 0.125 μCi/μmol). cAMP level was determined in aliquots of cell incubations, taken 4 minutes after addition of either glibenclamide, meglitinide, or vehicle, using a radioimmunologic method (cAMP [^{125}I]RIA Kit; Du Pont de Nemours, Bad Homburg, Germany).[22] Protein was assayed by the method of Lowry et al,[23] using bovine serum albumin as standard; 1 g packed hepatocytes corresponded to 220 ± 5 mg protein.

Enzymatic Assays

Total glycogen phosphorylase and glycogen phosphorylase *a* were assayed in 0.2-mL aliquots of cell suspensions taken at the indicated times, as described elsewhere[24]; 6-phosphofructo-2-kinase activity was measured as indicated by Bartrons et al.[25] The active form, as well as total activity, of fructose-2,6-bisphosphatase were measured by the disappearance of F-2,6-P_2 using basically the method described by Van Schaftingen et al[26] with some modifications. At selected times, 2-mL aliquots of the cell incubations were taken. After centrifugation (1,000 × *g* for 1 minute), cell pellets were homogenized with 3 vol of a medium containing 20 mmol/L glycerol-2-phosphate, 50 mmol/L HEPES, 100 mmol/L KCl, 1 mmol/L DTT, and 10 mmol/L EDTA, at pH 7.0. The homogenate was centrifuged at 10,000 × *g* for 15 minutes at 4°C, and 50-μL aliquots of the resulting supernatant were used to assay fructose-2,6-bisphosphatase activity at 37°C in a final volume of 250 μL. To assay the active form of the enzyme, the incubation medium contained 50 mmol/L HEPES, 100 mmol/L KCl, 3 mmol/L $MgCl_2$, 5 mmol/L glycerol-2-phosphate, 1 mmol/L ATP, 5 μmol/L F-2,6-P_2, and 0.5 mmol/L NADP, at pH 7.1. For the assay of total fructose-2,6-bisphosphatase activity, 50 mmol/L HEPES was replaced by 50 mmol/L Mes and the pH was adjusted to 5.8. At 0, 5, 10, and 20 minutes of incubation, 50-μL samples were taken and immediately pipetted into test tubes containing 150 μL 2-mmol/L NaOH. After a 30-fold dilution in distilled water, F-2,6-P_2 was assayed as indicated earlier. In some experiments, 6-phosphofructo-2-kinase and fructose 2,6-bisphosphatase activities were measured in 6% to 14% (wt/vol) polyethylene glycol-6000 fractions obtained from hepatocyte extracts.[27] The direct influence of meglitinide on these enzyme activities was investigated by incorporating this compound into the assay mixture.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined with Fura-2, basically using the method described by Grynkiewicz et al[28] as reported in detail elsewhere.[11] Briefly, loading of hepatocytes with Fura-2 was performed by incubating isolated liver cells (10^6/mL) for 30 minutes at 37°C with 5 μmol/L Fura-2 acetoxymethyl ester and 0.02% Pluronic F-127 in medium A containing 121 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4$, 2.5 mmol/L $CaCl_2$, 5 mmol/L $NaHCO_3$, 10 mmol/L HEPES, 10 mmol/L glucose, and 0.5% bovine serum albumin, at pH 7.4. Fluorescence of control and Fura-2–loaded hepatocytes was measured in a Perkin-Elmer model LS50 spectrofluorometer (Beaconsfield, Buckinghamshire, UK), and calculation of $[Ca^{2+}]_i$ was made by Intracellular Biochemistry Application Software (Perkin-Elmer). The dual excitation wavelengths were 340 and 361 nm (the isobestic point in our assay conditions); the emission wavelength was 510 nm. The K_d value corresponding to the Fura-2 · Ca^{2+} complex was 224 nmol/L.[28] For measurement of $[Ca^{2+}]_i$, hepatocytes ($\sim 10^5$ cells) were incubated in 2 mL medium A deprived of bovine serum albumin in a quartz cuvette at 33°C and kept in suspension by constant stirring. Maximal fluorescence of the Fura-2 · Ca^{2+} complex was obtained by lysing the cells with 0.2% sodium dodecyl sulfate. Then a mixture of EGTA/Tris, pH 8.0, was added (final concentrations in the cuvette, 10/60 mmol/L) to determine the minimal fluorescence. In experiments involving Ca^{2+} analysis, single registers are shown, but similar results were obtained in at least three separate experiments from independent cell preparations.

Statistical Analysis

Statistical significance of differences between values was calculated by the paired or unpaired Student's *t* test. Differences were considered statistically significant when *P* was less than .05. Half-maximally effective concentration (EC_{50}) values for glibenclamide and meglitinide were calculated using the Enzfitter program (Elsevier-Biosoft, Cambridge, UK).

RESULTS

Addition of glibenclamide (20 μmol/L) to hepatocyte suspensions caused a significant increase in the amount of the active form of glycogen phosphorylase present in the cells (Fig 2). This effect was clearly observed 2 and 4 minutes after sulfonylurea addition (~65% and 56% > control values, respectively), with the activation being progressively reduced in the following minutes. The presence of meglitinide (50 μmol/L) in the incubation medium also activated glycogen phosphorylase (~60% and 38% > corresponding control values at 2 and 4 minutes of incubation, respectively), although in this case the effect seemed more transient (Fig 2). Simultaneously with the activation of glycogen phosphorylase, glibenclamide caused a significant increase in hepatocyte content of F-2,6-P_2, which was approximately 48% greater than the control value after a 20-minute incubation (19.2 ± 1.1 *v* 12.9 ± 0.7 nmol/g cells, n = 4, $P < .001$). In contrast, meglitinide significantly reduced hepatocyte F-2,6-P_2 levels (~23% after a 20-minute incubation; Fig 2).

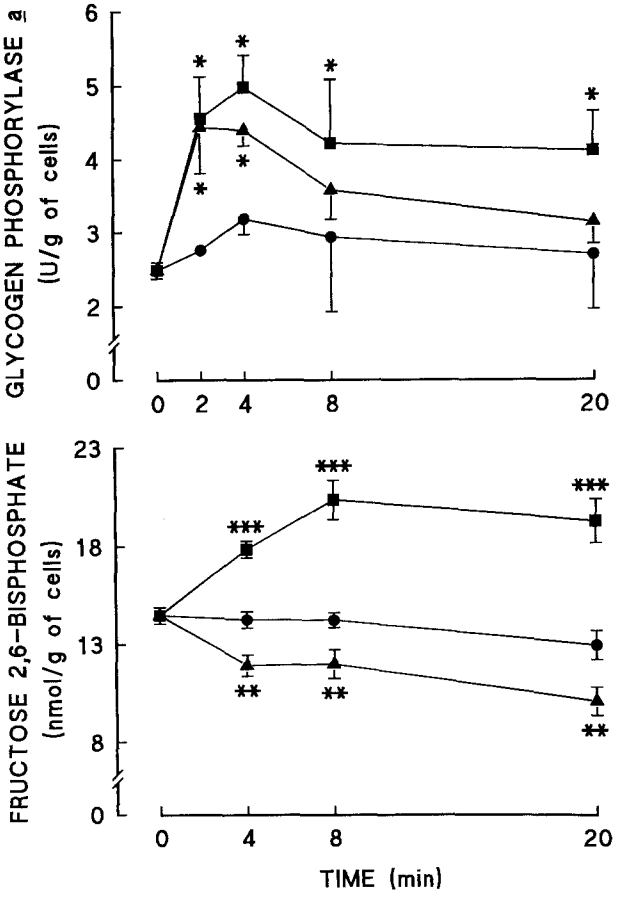


Fig 2. Time course of the effect of glibenclamide and meglitinide on glycogen phosphorylase *a* activity and on F-2,6-P_2 level in isolated rat hepatocytes. Cells were preincubated in Krebs-Henseleit medium with 10 mmol/L glucose for 30 minutes. Then 20 μmol/L glibenclamide (■), 50 μmol/L meglitinide (▲), or vehicle (●) was added to cell incubations. Afterward, aliquots were taken at the indicated times for assay of glycogen phosphorylase *a* activity or F-2,6-P_2. Mean ± SEM of 4 experiments. Paired Student's *t* test: *$P < .05$, **$P < .01$, and ***$P < .001$ *v* corresponding vehicle incubations.

Modulation of both glycogen phosphorylase *a* activity and F-2,6-P_2 level by glibenclamide was dose-dependent (Fig 3); calculated EC_{50} values were 3.7 ± 1.6 and 6.8 ± 2.5 μmol/L (n = 4 to 5), respectively. Similarly, both the activation of glycogen phosphorylase and reduction of cellular levels of F-2,6-P_2 elicited by meglitinide were also dose-dependent, with calculated EC_{50} values being 9.6 ± 3.3 and 1.9 ± 0.2 μmol/L (n = 5), respectively (Fig 3).

Cellular concentrations of adenine nucleotides were not significantly modified by the presence of either glibenclamide or meglitinide in the incubation medium, indicating that the changes observed in glycogen phosphorylase *a* activity and F-2,6-P_2 level were not the consequence of a nonspecific toxic effect of these agents on the energetic metabolism of hepatocytes (control hepatocytes: ATP 2.60 ± 0.41 μmol/g cells, ADP 0.57 ± 0.12, AMP 0.08 ± 0.03; glibenclamide-treated hepatocytes: ATP 2.7 ± 0.37, ADP 0.51 ± 0.13, AMP 0.07 ± 0.02; meglitinide-treated hepatocytes: ATP 2.61 ± 0.40, ADP 0.53 ± 0.08, AMP 0.08 ± 0.04). Values represent the mean ± SEM of four experiments; adenine nucleotide levels were measured in aliquots of cell suspensions taken after 4 minutes of incubation. Meglitinide—as previously demonstrated for glibenclamide[11]—did not significantly modify cellular levels of cAMP (0.33 ± 0.06, 0.28 ± 0.09, and 0.29 ± 0.09 nmol/g cells, respectively, for control, glibenclamide-, and meglitinide-treated hepatocytes; n = 4 experiments). These findings, together with the fact that activation of glycogen phosphorylase caused by meglitinide was markedly reduced in hepatocytes incubated in Ca^{2+}-free Krebs-Henseleit medium in the presence of 2 mmol/L EGTA (Table 1), support the implication of a Ca^{2+}-dependent mechanism in the activation of glycogen phosphorylase by this agent. In connection with this, Fig 4 shows that meglitinide (50 μmol/L) caused a significant increase in hepatocyte $[Ca^{2+}]_i$, similar to that caused by an identical concentration of glibenclamide. The calculated concentration of meglitinide responsible for the half-maximal effect on hepatocyte $[Ca^{2+}]_i$ corresponded to 12 μmol/L (data not shown). As a comparison, the influence of a saturating concentration of the α-adrenergic agonist phenylephrine (10 μmol/L) on both glycogen phosphorylase *a* activity and $[Ca^{2+}]_i$ was also studied. As shown in Table 1, activation of glycogen phosphorylase by phenylephrine was almost maximal in hepatocytes incubated in the presence of Ca^{2+}, and was significantly reduced in the absence of this cation, as previously reported.[29] As expected, phenylephrine caused a marked increase in $[Ca^{2+}]_i$ in Fura-2–loaded hepatocytes (Fig 4).

The increase in hepatocyte F-2,6-P_2 levels caused by glibenclamide was associated with a partial blockade in the rate of glucose formation from a mixture of [^{14}C]lactate/pyruvate 4/0.4 mmol/L (Table 2). Under similar conditions, the reduction in F-2,6-P_2 levels caused by meglitinide was accompanied by a significant acceleration of the gluconeogenic flux. Furthermore, a significant inverse correlation could be established between the rate of gluconeogenesis and the cellular concentration of F-2,6-P_2 when individual data included in Table 2 were plotted together

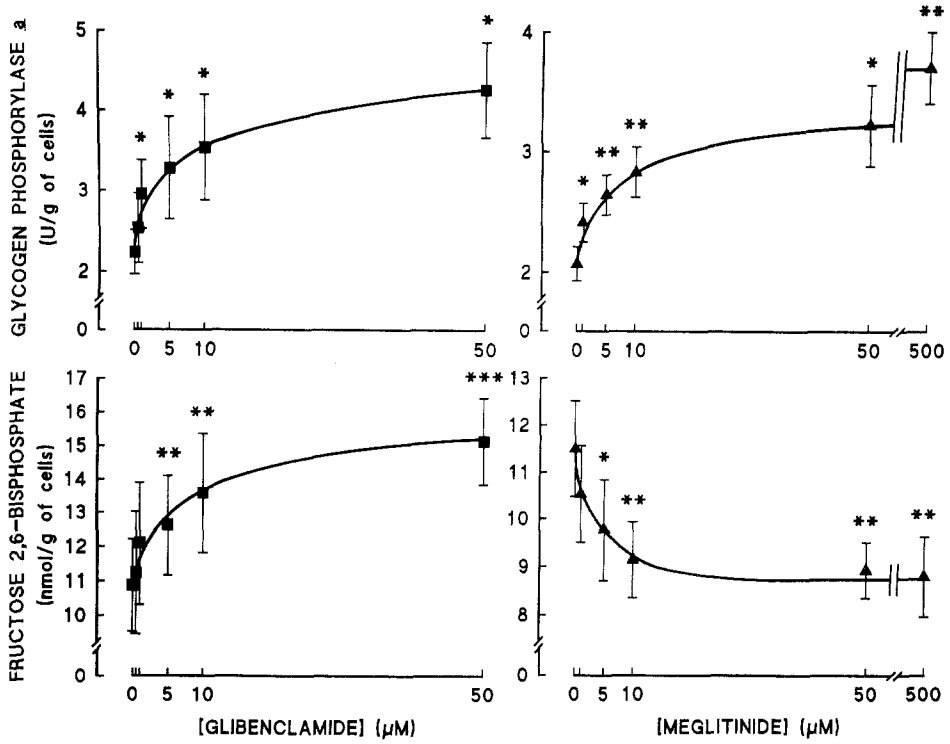


Fig 3. Effect of different concentrations of either glibenclamide or meglitinide on glycogen phosphorylase *a* activity and F-2,6-P_2 level in isolated rat hepatocytes. Mean ± SEM of 4 or 5 experiments. Paired Student's *t* test: *$P < .05$, **$P < .01$, and ***$P < .001$ *v* corresponding vehicle incubations.

($r = .724$, $P < .05$, n = 9). This close inverse correlation supports a fundamental role of F-2,6-P_2 levels in the control of hepatocyte gluconeogenesis under the assayed experimental conditions.

To investigate the mechanism by which glibenclamide and meglitinide modulate the cellular concentration of F-2,6-P_2, we studied the influence of these two agents on the level of fructose-6-phosphate, as well as on both 6-phosphofructo-2-kinase and fructose-2,6-bisphosphatase activities. In good agreement with the reported glycogenolytic effect, glibenclamide (20 μmol/L) significantly increased hepatocyte content of fructose-6-phosphate measured in aliquots of cell incubations taken 8 minutes after sulfonylurea addition (Table 3). Under similar conditions, meglitinide (50 μmol/L) did not significantly affect fructose-6-phosphate concentration; this could be due to the more transient activation of glycogen phosphorylase by meglitinide (Fig 2). In Table 3 it is also shown that the amount of 6-phosphofructo-2-kinase in active form present in hepatocytes was not significantly modified by addition of either glibenclamide or meglitinide to the incubation medium. When the influence of these two agents on hepatocyte fructose 2,6-bisphosphatase activity was examined (Table 4), it was observed that meglitinide clearly increased the percentage of enzyme in active form without affecting the total activity. In contrast, addition of glibenclamide to

Table 1. Effect of Glibenclamide, Meglitinide, and Phenylephrine on Glycogen Phosphorylase *a* Activity in Isolated Rat Hepatocytes Incubated in the Presence or Absence of Calcium

Additions	Glycogen Phosphorylase *a* (% of total)	
	With Ca^{2+}	Without Ca^{2+}
Vehicle	37.5 ± 2.6	30.2 ± 3.3
Glibenclamide 20 μmol/L	50.7 ± 4.6†	37.7 ± 6.4 (NS)
Meglitinide 50 μmol/L	45.0 ± 4.3*	36.0 ± 5.2 (NS)
Phenylephrine 10 μmol/L	99.2 ± 7.1†	70.5 ± 5.2†

NOTE. Mean ± SEM of 4 experiments.

Paired Student's *t* test *v* corresponding vehicle incubation: *$P < .05$; †$P < .01$; NS, not significant.

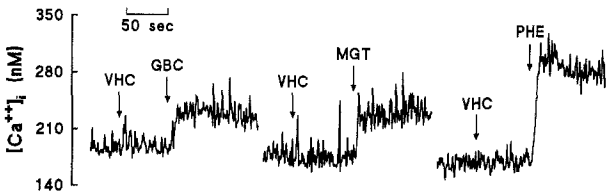


Fig 4. Effect of glibenclamide (GBC), meglitinide (MGT), and phenylephrine (PHE) on $[Ca^{2+}]_i$ in isolated rat hepatocytes. Arrows indicate the moment of vehicle (VHC), GBC (50 μmol/L), MGT (50 μmol/L), or PHE (10 μmol/L) addition. Data are from a representative experiment. Basal level of hepatocyte $[Ca^{2+}]_i$ measured in a series of experiments corresponded to 141 ± 21 nmol/L (n = 20 experiments). Mean maximal increases of hepatocyte $[Ca^{2+}]_i$ in response to GBC, MGT, and PHE were 48 ± 9, 45 ± 7, and 191 ± 15 nmol/L (n = 5 to 8 experiments).

Table 2. Effect of Glibenclamide and Meglitinide on Gluconeogenesis and F-2,6-P_2 Levels in Isolated Rat Hepatocytes

Additions	[U-^{14}C]lactate Converted to Glucose (μmol/g cells × 30 min)	F-2,6-P_2 (nmol/g cells)
Vehicle	2.9 ± 0.1	6.3 ± 0.8
Glibenclamide 20 μmol/L	1.9 ± 0.3*	7.8 ± 0.9*
Meglitinide 50 μmol/L	4.2 ± 0.1*	3.6 ± 1.3*

NOTE. Mean ± SEM of 3 experiments.

Paired Student's *t* test *v* corresponding vehicle incubation: *$P < .05$.

Table 3. Effect of Glibenclamide and Meglitinide on the Concentration of Fructose-6-Phosphate (n = 3) and on 6-Phosphofructo-2-kinase Activity (n = 4) in Isolated Rat Hepatocytes

Additions	Fructose-6-Phosphate (nmol/g cells)	6-Phosphofructo-2-kinase, Active Form (mU/g cells)
Vehicle	98.6 ± 5.2	11.1 ± 1.0
Glibenclamide 20 μmol/L	135.1 ± 7.3*	13.3 ± 0.9(NS)
Meglitinide 50 μmol/L	94.8 ± 8.3 (NS)	12.2 ± 0.8(NS)

NOTE. Mean ± SEM of 3 or 4 different experiments.

Paired Student's *t* test *v* corresponding vehicle incubation: *$P < .05$; NS, not significant.

hepatocyte suspensions did not significantly modify either the total activity or the percentage in active form of fructose-2,6-bisphosphatase.

We also studied the influence of different concentrations of meglitinide on both 6-phosphofructo-2-kinase and fructose-2,6-bisphosphatase activities, measured in a partially purified enzyme preparation obtained by polyethylene glycol fractionation (6% to 14% wt/vol) of hepatocyte extracts. Under these conditions, the presence of meglitinide in the assay mixture did not significantly affect 6-phosphofructo-2-kinase activity (data not shown). In contrast, this compound stimulated the active form of fructose-2,6-bisphosphatase in a dose-dependent manner, attaining a maximal effect of approximately 40% over the basal value; the calculated ED_{50} of meglitinide was 1.7 μmol/L (Fig 5). It must also be mentioned that under similar assay conditions, meglitinide did not affect the rate of hydrolysis of *p*-nitrophenyl phosphate (10 mmol/L) by a nonspecific phosphatase activity present in the polyethylene glycol extracts (data not shown).

DISCUSSION

Sulfonylureas stimulate insulin secretion by blocking ATP-dependent K^+ channels in the plasma membrane of pancreatic β cells; the resultant depolarization leads to the opening of voltage-dependent Ca^{2+} channels and allows Ca^{2+} influx.[15,16,30,31] For a long time, it was thought that the sulfonylurea group was an essential structural requirement for the insulin-releasing effect. However, in vivo[13] and in vitro[14] studies have demonstrated that acyl-amino-alkyl benzoic acid derivatives such as meglitinide, which is similar to the nonsulfonylurea moiety of glibenclamide, also stimulate insulin secretion. Experimental evidence indicates that both glibenclamide and meglitinide accelerate the liberation of insulin basically by the same molecular mechanism.[14-16] However, when assayed at nonsaturating concentrations, meglitinide showed a markedly lower efficacy (~100 to 200-fold) than glibenclamide, a finding that has been directly correlated with their different K^+-channel–blocking potencies.[14,32]

Table 4. Effect of Glibenclamide and Meglitinide on Fructose-2,6-Bisphosphatase Activity in Isolated Rat Hepatocytes

Additions	Fructose-2,6-bisphosphate	
	Total Activity (mU/g cells)	Active Form (% of total)
Vehicle	13.5 ± 1.4	10.8 ± 1.1
Glibenclamide 50 μmol/L	12.8 ± 0.9(NS)	13.5 ± 0.8 (NS)
Meglitinide 100 μmol/L	13.0 ± 1.5(NS)	17.0 ± 1.5*

NOTE. Mean ± SEM of 3 different experiments.

Paired Student's *t* test *v* corresponding vehicle incubation: *$P < .05$; NS, not significant.

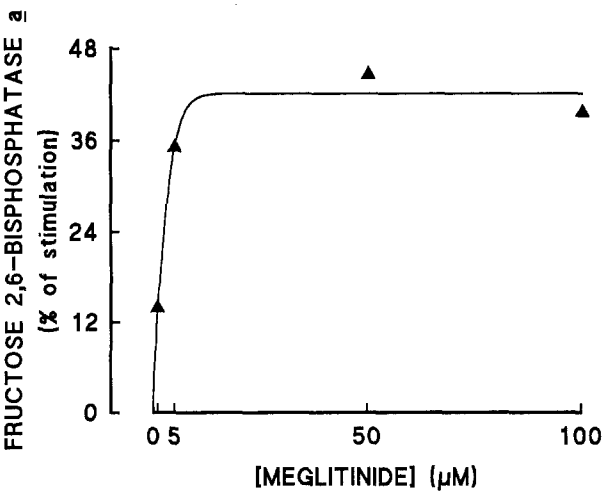


Fig 5. Stimulatory effect of meglitinide on the active form of fructose-2,6-bisphosphatase. Data are the mean of two experiments performed in duplicate.

With respect to the hypoglycemic effect of sulfonylureas, many reports have suggested that extrapancreatic actions of these drugs could also collaborate in the control of glycemia.[33,34] Among them, reduction of hepatic glucose output could play a relevant role.[35,36] In connection with this, experiments performed in perfused livers,[37,38] hepatic explants,[39] or isolated rat hepatocytes[4,6,7] have demonstrated that sulfonylureas may inhibit hepatic gluconeogenesis from different precursors. Moreover, in hepatocytes isolated from fed rats, a close correlation has been established between the inhibition of gluconeogenesis and the increase in F-2,6-P_2 levels caused by sulfonylureas.[4,6,7]

Although the mechanism by which sulfonylureas increase F-2,6-P_2 levels is not clearly established, it seems that a paradoxic glycogenolytic effect elicited by these agents could collaborate in elevating the hepatocyte content of F-2,6-P_2 in the fed state.[10] In fact, sulfonylureas have been demonstrated to increase $[Ca^{2+}]_i$ in isolated rat hepatocytes, with a subsequent Ca^{2+}-dependent activation of glycogen phosphorylase.[11]

In this study, we have examined the effects of both glibenclamide and meglitinide on hepatocyte glycogen phosphorylase *a* activity, F-2,6-P_2 level, and $[Ca^{2+}]_i$ to elucidate which among the hepatic effects of glibenclamide are exerted by the sulfonylurea moiety.

Our results demonstrate that meglitinide, structurally related to the nonsulfonylurea moiety of glibenclamide, was able to activate hepatocyte glycogen phosphorylase in a dose-dependent manner. As previously demonstrated for

glibenclamide,[11] this activation occurred without significant changes in cellular levels of cAMP and required the presence of Ca^{2+} in the incubation medium. Moreover, meglitinide—similar to glibenclamide—caused a small, dose-dependent increase in hepatocyte $[Ca^{2+}]_i$. The mechanism by which glibenclamide and meglitinide mediate the increase in hepatocyte $[Ca^{2+}]_i$ is unknown. The lack of blockade of the effect of glibenclamide by either nifedipine or verapamil[11] rules out the participation of voltage-operated Ca^{2+} channels in liver cells, in contrast to what has been described in pancreatic β cells.[15,16,30,31] Moreover, ATP-dependent K^+ channels have not been identified in the plasma membrane of hepatocytes.[15,16,40] It can be speculated that both glibenclamide and meglitinide act on liver cells through interaction with low-affinity binding sites able to modulate Ca^{2+} fluxes. However, their presence in hepatocytes has not yet been demonstrated. It is of note that the calculated EC_{50} values for both meglitinide and glibenclamide as activators of glycogen phosphorylase were in the low micromolar range (9.6 ± 3.3 and 3.7 ± 1.6 μmol/L, respectively). This is in good agreement with the apparent activation constant (K_a) values reported for glibenclamide in potentiating insulin effects on either 3-*O*-methyl-D-glucose transport in rat adipocytes (K_a, 1 μmol/L)[41] or glycogen synthase activation in rat adipose tissue (K_a, 2 μmol/L),[42] as well as in stimulating [^{3}H]-2-deoxyglucose uptake in BC3H1 myocytes (K_a, ~2 μmol/L).[43] However, when EC_{50} values for meglitinide and glibenclamide obtained in hepatocytes were compared with those reported for these two agents as stimulators of insulin secretion (1.5 μmol/L and 0.5 nmol/L, respectively),[32] a close correspondence was observed in the case of meglitinide, but a discrepancy of approximately three orders of magnitude was evidenced for glibenclamide. This could be explained by the presence of high-affinity (K_d, 0.4 to 1 nmol/L) and low-affinity (K_d, 10 to 300 nmol/L) binding sites for sulfonylureas in pancreatic β cells.[15,16,40] In these cells, glibenclamide would stimulate insulin secretion by interacting with high-affinity sites,[15,16] and the effects of meglitinide would be exerted through low-affinity binding sites.[44,45]

Another possible mechanism of action of sulfonylureas on the liver could be related to the fact that these agents are able to bind to artificial phospholipid bilayers in the low-micromolar range.[46] In this respect, a direct and saturable interaction between sulfonylureas and certain types of phospholipids of the hepatocyte plasma membrane could lead to an increased Ca^{2+} permeability.[47]

As a result of its glycogenolytic action, glibenclamide significantly increased the cellular concentration of fructose-6-phosphate, cooperating in this way to increase hepatocyte content of F-2,6-P_2. In contrast, the more transient activation of glycogen phosphorylation caused by meglitinide was not accompanied by a significant change in fructose-6-phosphate concentration measured after 8 minutes of incubation. Moreover, meglitinide provoked a significant dose-dependent reduction in F-2,6-P_2 levels, and—without affecting 6-phosphofructo-2-kinase activity—caused an apparent increase in the percentage of fructose-2,6-bisphosphatase in active form. It must be mentioned that 6-phosphofructo-2-kinase and fructose-2,6-bisphosphatase activities are located in the same protein, being reciprocally regulated by phosphorylation/dephosphorylation mechanisms.[9] Thus, phosphorylation of the protein by cAMP-dependent protein kinase causes inactivation of the kinase and provokes activation of the phosphatase, and dephosphorylation by protein phosphatases reverses these effects.[9] However, the lack of reciprocal changes in 6-phosphofructo-2-kinase and fructose-2,6-bisphosphatase activities does not support the hypothesis that meglitinide affects the phosphorylation state of the protein. This concept is further reinforced by the observation that meglitinide stimulates fructose-2,6-bisphosphatase activity in a partially purified enzyme preparation without affecting kinase activity. All these findings suggest that activation of fructose-2,6-bisphosphatase by meglitinide results from a direct modulation of bisphosphatase activity. In intact hepatocytes, this interaction would be favored by the lipophilicity of meglitinide, which allows the drug to penetrate into the cell.[32] To support further the close relationship between the reduction in hepatocyte F-2,6-P_2 levels and stimulation of fructose-2,6-bisphosphatase, the calculated EC_{50} values of meglitinide for these two processes were similar (1.9 and 1.7 μmol/L, respectively).

As a consequence of the reduction in hepatocyte F-2,6-P_2 levels, meglitinide stimulated the gluconeogenic flux from [^{14}C]lactate/pyruvate in hepatocytes isolated from fed rats. Under these metabolic conditions, changes in the cellular concentration of F-2,6-P_2 are known to play a regulatory role in the control of hepatic gluconeogenesis.[9,25] By contrast, and in agreement with a more general effect of sulfonylureas,[4,6,7] the increase in F-2,6-P_2 levels caused by glibenclamide was accompanied by inhibition of hepatocyte glucose production from lactate. It is of note that intravenous administration of meglitinide to dogs caused a marked hypoglycemia,[13] indicating that in vivo the effect on insulin secretion predominates over the direct effect of this agent on hepatic gluconeogenesis.

In summarizing our results, we can say that in isolated rat hepatocytes, the nonsulfonylurea moiety of glibenclamide—structurally related to meglitinide—appears to be responsible for the activation of glycogen phosphorylase by a Ca^{2+}-dependent mechanism. With respect to the modulation of F-2,6-P_2 levels, meglitinide causes a significant reduction in the concentration of this regulatory metabolite by stimulating fructose-2,6-bisphosphatase. The sulfonylurea moiety of glibenclamide seems to hamper bisphosphatase stimulation, allowing glibenclamide to increase hepatocyte F-2,6-P_2 levels at least in part as a result of its glycogenolytic effect.

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