Stimulation of Insulin Release by Repaglinide and Glibenclamide Involves Both Common and Distinct Processes

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The action of repaglinide, a novel insulin secretagogue, was compared with the sulfonylurea glibenclamide with regard to the hypoglycemic action in vivo, binding to βTC-3 cells, insulin secretion from perifused mouse islets, and capacity to stimulate exocytosis by direct interaction with the secretory machinery in single voltage-clamped mouse β-cells. Two binding sites were identified: a high-affinity repaglinide (K_D = 3.6 nmol/l) site having lower affinity for glibenclamide (14.4 nmol/l) and one high-affinity glibenclamide (25 nmol/l) site having lower affinity for repaglinide (550 nmol/l). In contrast to glibenclamide, repaglinide (in concentrations as high as 5 µmol/l) lacked the ability to enhance exocytosis in voltage-clamped β-cells. Repaglinide was more potent than glibenclamide in stimulating insulin release from perifused mouse islets (EC_{50} 29 vs. 80 nmol/l). The greater potency of repaglinide in vitro was paralleled by similar actions in vivo. The ED_{50} values for the hypoglycemic action were determined to be 10.4 and 15.6 µg/kg after intravenous and oral administration, respectively. The corresponding values for glibenclamide were 70.3 µg/kg (intravenous) and 203.2 µg/kg (oral). Further, repaglinide (1 mg/kg p.o.) was effective (P < 0.001) as an insulin-releasing agent in a rat model (low-dose streptozotocin) of type 2 diabetes. These observations suggest that the insulinotropic actions of repaglinide and glibenclamide in vitro and in vivo are secondary to their binding to the high-affinity repaglinide site and that the insulinotropic action of repaglinide involves both distinct and common cellular mechanisms. ***Diabetes*** **47:345–351, 1998**

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H.K. and P.M. hold stock in Novo Nordisk.

HSA, human serum albumin; PPP, (+)-3-(3-hydroxyphenyl)-*N*-(1-propyl)-piperidine; STZ, streptozotocin; SU, sulfonylurea; SUR, sulfonylurea receptor.

Insulin secretion is controlled by the membrane potential of the β-cell, which depends on the activity of ATP-sensitive K^+ channels (K_{ATP} channels) in the plasma membrane (1). The K_{ATP} channels close following an increase in the cytoplasmic ATP/ADP ratio. This leads to membrane depolarization, opening of voltage-gated Ca^{2+} channels, elevation of the cytoplasmic Ca^{2+} concentration, and stimulation of Ca^{2+}-dependent exocytosis of the insulin-containing granules. The K_{ATP} channels represent a target for the hypoglycemic sulfonylureas (SUs; 1), a group of compounds that has been successfully used in the treatment of type 2 diabetes for more than 30 years. Recently, it was demonstrated that the K_{ATP} channel is a complex of a 140-kDa sulfonylurea receptor (SUR) and an inward rectifier channel protein (KIR6.2) (2).

Treatment of type 2 diabetes with SUs is associated with a number of problems. These include hypoglycemic episodes, secondary failure, and possible cardiovascular side effects (3). In attempts to overcome these problems, several novel antidiabetic compounds are currently in development, among them repaglinide, (S)-(+)-2-ethoxy-4[2-[[3-methyl-1-[2-(1-piperidinyl)-phenyl]-butyl]amino]-2-oxo-ethyl] benzoic acid (4) (AG-EE 623 ZW), which is structurally distinct from the traditional SUs but shows some chemical resemblance to the nonsulfonylurea moiety of the glibenclamide molecule (Fig. 1). The present study was initiated to compare the mechanism of action of repaglinide with that of glibenclamide in vitro and in vivo.

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RESEARCH DESIGN AND METHODS

Repaglinide (batches 803099 and 8230301) was synthesized and supplied by Dr. Karl Thomae GmbH (Biberach, Germany). Both tritiated repaglinide and protein A scintillation proximity assay particles were purchased from Amersham (Buckinghamshire, U.K.). Radiolabeled (^{3}H) glibenclamide was obtained from DuPont (Boston, MA), and unlabeled glibenclamide was from Sigma (St. Louis, MO). Human serum albumin (HSA) was from Behringwerke (Marburg, Germany), and collagenase A was from Boehringer Mannheim (Mannheim, Germany).

The protein assay kit was from Pierce (Rockford, IL). Culture flasks and petri dishes were obtained from Nunc (Roskilde, Denmark). Four-well plates were from Linbro (ICN, Costa Mesa, CA), and media for cell culture were supplied by Gibco (Grand Island, NY) or Life Technologies (Paisley, U.K.).

Repaglinide and glibenclamide were prepared as concentrated (20 mmol/l) stock solutions using DMSO that was subsequently diluted to the desired concentrations; the final concentration of DMSO was 0.5% (vol/vol).

Ligand binding experiments. Binding of glibenclamide and repaglinide was assayed using monolayers of βTC3 insulinoma cells (5) obtained from Cold Spring Harbor Laboratory (Cold Spring Harbor, NY). Two days before the experiments, the cells were plated into four-well tissue culture plates (28.3 cm^2) in Dul-

becco's modified Eagle's medium (5.5 mmol/l D-glucose, Gibco); supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin; and incubated in 5% CO_2 in humidified air at 37°C. The monolayers (~10^7 cells per well) were then washed at 5°C in a medium consisting of 10 mmol/l HEPES, 130 mmol/l NaCl, 4.7 mmol/l KCl, 1.4 mmol/l $MgSO_4$, 2.5 mmol/l NaH_2PO_4, 3 mmol/l D-glucose, and 1 mmol/l EGTA (pH 7.4), and were supplemented with 2 g/l HSA and 8×10^8 IU/l aprotinin. The cells were then incubated for 2 h with 20 pmol/l [^{3}H]repaglinide (specific activity 49 Ci/mmol, 1 mCi/ml) or 20 pmol/l [^{3}H]glibenclamide (specific activity 48.5 Ci/mmol, 1 mCi/ml) and unlabeled test compounds at 5°C in a total volume of 3 ml. After incubation, cells were rinsed once with 4 ml of ice-cold buffer and three times further with a modified buffer (4 ml) lacking HSA and aprotinin. Cells were then lysed with NaOH (2 ml, 0.1 mmol/l) for 30 min, and the lysates were transferred to 18 ml Ultima Gold scintillation cocktail (Packard, Meriden, CT) in glass vials for assessment of bound radioactivity using a Packard Tricarb β-counter. The amount of protein was assayed in unlabeled monolayers (range of 1.0–1.5 mg), and binding of the compounds was expressed as picomoles per milligram protein. All determinations were made in duplicate.

In saturation experiments, nonspecific binding was determined in the presence of 10 µmol/l unlabeled repaglinide or glibenclamide. For the competition experiments, nonspecific binding was determined using 0.02 nmol/l [^{3}H]repaglinide or [^{3}H]glibenclamide in the presence of 1 µmol/l repaglinide or 1 µmol/l glibenclamide, respectively, and amounted to 28 ± 2% (n = 6) and 20 ± 2% (n = 6) of total binding, respectively.

The experiments on glibenclamide and repaglinide binding were conducted in parallel and on the same passage and number of cells.

Insulin release. Insulin secretion was studied in vitro using intact mouse pancreatic islets isolated from adult male NMRI mice (Gl. Bomholtgaard, Ry, Denmark). The islets were isolated by collagenase digestion as previously described (6) and kept in tissue culture overnight in 5 ml RPMI 1640 medium supplemented with 10% newborn calf serum (Gibco), 20 mmol/l HEPES (pH 7.4), 5 mmol/l $NaHCO_3$, 100 U/ml penicillin, and 100 µg/ml streptomycin. The dynamics of insulin secretion were investigated in a Brandel Superfusion 2000 computer-controlled perifusion system essentially as described previously (7). Perifusion buffer consisted of 20 mmol/l HEPES, 115 mmol/l NaCl, 5 mmol/l $NaHCO_3$, 4.7 mmol/l KCl, 2.6 mmol/l $CaCl_2$, 2 mmol/l glutamine, 1.2 mmol/l KH_2PO_4, and 1.2 mmol/l $MgSO_4$ (pH 7.4) supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 2 g/l HAS, and D-glucose as indicated in the text and figure legends. In each experiment, 30 islets were added in buffer to the top of a column consisting of 300 µl Bio-Gel P-2 beads (Bio-Rad Laboratories, Richmond, CA) and carefully mixed with the beads. The islets were perifused at a flow rate of 0.30 ml/min. The rate of insulin release is expressed as picomoles per minute per 100 islets. Islets were first perifused for 60 min at 3 mmol/l D-glucose to obtain a basal insulin release rate and then perifused for a further 50 min with various concentrations of D-glucose before repaglinide or glibenclamide was added. The secretory rate during the last 10 min before the addition of the drugs was used as the reference level. After 30 min of stimulation with repaglinide or glibenclamide, the compounds were removed and the islets exposed to glucose alone for the last 40 min of the experiments. The effects of different concentrations of repaglinide and glibenclamide on insulin release were examined at 5 and 10 mmol/l D-glucose. The influence of the sugar concentration on the stimulatory action of the drugs was analyzed at concentrations of repaglinide and glibenclamide that were roughly equipotent (40 nmol/l for repaglinide and 200 nmol/l for glibenclamide).

The amount of insulin released was determined by scintillation proximity assay (8) using guinea pig anti-insulin serum (PNILGP4), mono-^{125}I-[TyrA14] human insulin (Novo Nordisk A/S, Bagsværd, Denmark) as a tracer, and rat insulin (Novo Nordisk A/S) as the standard.

Electrophysiological measurements. The electrophysiological experiments were carried out on single mouse pancreatic β-cells, prepared and maintained in tissue culture for up to 3 days in RPMI 1640 medium, as described elsewhere (9). The effects of glibenclamide and repaglinide on exocytosis of the secretory granules were investigated using measurements of cell capacitance as an indicator (10).

In vivo studies

Animals. Adult male Sprague-Dawley rats (Møllegård Breeding Centre, Ll. Skensved, Denmark) were acclimatized for at least 4 days before use. They were kept in controlled housing conditions on a 12:12 h light:dark cycle, fed standard rat food (Altromin Diet No 1324, C. Petersen A/S, Ringsted, Denmark), and allowed access to drinking water ad libitum. Animals within the body weight range 160–250 g were used in the study.

Measurements of oral potency of compounds. Repaglinide and glibenclamide were each dissolved in a vehicle consisting of 2% Tween-80 (Merck, Darmstadt, Germany) in 0.9% saline and were administered by gavage using a dose volume of 2.5 ml/kg. Animals were allocated into three groups of six individuals receiving vehicle alone, repaglinide (0.003–1.0 mg/kg), or glibenclamide (0.03–10 mg/kg). Blood glucose levels were determined by enzymatic analysis (EBIO 6666 autoanalyser, Eppendorff, Hamburg, Germany) of blood samples (10 µl) drawn from the tail tip immediately before or 120 min after treatment. It was ascertained that the latter time point is appropriate to observe maximal effect.

Determination of intravenous potency of compounds. Repaglinide and glibenclamide were prepared as stock solution (10 mg/ml) in DMSO. The drugs were added to final concentration in a 1:1 mixture of phosphate-buffered saline and 0.9% saline. The volume of the administered dose was 4 ml/kg. The highest concentration of DMSO in the injected solution was 2.5%, and the control solution was prepared with the same amount of the solvent.

Rats were allocated into groups of 4–7 individuals and anesthetized as described (11). A cannula (Venflon 2, 22G; Viggo-Spectramed, Helsingborg, Sweden) was inserted in a lateral tail vein of each animal for intravenous injection of either repaglinide (0.003–1.0 mg/kg) or glibenclamide (0.003–1.0 mg/kg). Blood samples were taken immediately before and 45 min after injection of the drug, and the percentage reduction of the blood glucose concentration was determined.

Method for inducing moderate diabetes with low-dose streptozotocin. Type 2–like diabetes was induced in CO_2-sedated, overnight-fasted rats by injection of streptozotocin (35 mg/kg) into a tail vein essentially as described previously (11). Rats with blood glucose concentrations >9 mmol/l were considered diabetic. Diabetic animals were divided into two groups of six individuals and fasted for 18 h before oral dosing with either vehicle (2% Tween-80 in 0.9% saline) or repaglinide (1 mg/kg). Plasma insulin and glucose concentrations were determined, using samples obtained from the orbital sinus under light CO_2 sedation, immediately before and 60 min after oral administration of repaglinide or vehicle. The blood glucose concentration was determined as described above. Plasma insulin level was determined using a rat insulin immunoassay kit (Novo Nordisk A/S) consisting of antibody M8170, A19 ^{125}I-labeled rat insulin as a tracer and a 1:2 mixture of rat insulin I and rat insulin II as a standard.

Statistical analysis. Results of data analysis are presented as mean values ± SE for the indicated number of experiments. Scatchard analysis of data from saturation experiments was performed using EBDA software (12). The specific binding data in the saturation experiments were fitted to a hyperbolic function and were drawn using SigmaPlot software (Jandel GmbH, Erkath, Germany) using values of K_D and B_{max} derived from the EBDA program. The nonspecific binding data were fitted using linear regression analysis. Analysis of the raw data from single-day competition assays was performed by fitting the data to single-site and two-site models to obtain the best estimates of binding affinities, using standard nonlinear methods (13). In Fig. 3, curves were fitted by nonlinear regression and drawn using the GraphPad Prism version 1.03 (GraphPad Software, San Diego, CA) in single- or two-site models depending on the result from the raw data analysis described above.

The stimulatory action of each of the drugs on insulin secretion was evaluated using the Student's t test for paired data using the perifusion fraction of 100–110 min as a reference value (Figs. 4 and 5). Comparison of effects of the two drugs on in vitro insulin secretion was performed with the Wilcoxon rank-order sum test for unpaired data.

The hypoglycemic action of the drugs is presented as the percentage reduction of the blood glucose concentration. ED_{50} values (and 95% CIs) were calculated by fitting a curve using nonlinear regression to the percentage changes of the blood glucose concentration. The slope of the linear regression was set to unity, and the minimum value was fixed at zero.

Statistical significance of the differences between repaglinide and glibenclamide in the in vivo experiments was calculated by comparison of means in a normally distributed population (U test). Basal fasting glucose levels in the mildly diabetic rats were compared by ANOVA, and the effect of repaglinide and vehicle on glycemia and insulin levels were compared using Student's t test.

RESULTS

Binding experiments to intact βTC3 cells using [^{3}H]repaglinide and [^{3}H]glibenclamide. Scatchard analysis of data from saturation experiments using [^{3}H]repaglinide revealed a single binding site with an estimated K_D of 6.4 ± 0.7 nmol/l (n = 4), a Hill coefficient (n_H) of 0.99 ± 0.01, and a B_{max} of 395 fmol/mg protein. Similar experiments with glibenclamide likewise suggested a single binding site. The values of K_D, n_H, and B_{max} were determined to be 23 ± 6 nmol/l, 1.00 ± 0.003, and 1.32 pmol/mg protein, respectively (n = 4). Data from single representative experiments are shown in Fig. 2.

Fig. 3*A* shows the results from competition assays using [^{3}H]repaglinide (upper panel) and [^{3}H]glibenclamide (lower panel). Using labeled repaglinide, Scatchard analysis (not shown) of data from competition assays indicated the presence of one binding site for repaglinide (K_D 3.6 ± 0.4 nmol/l;

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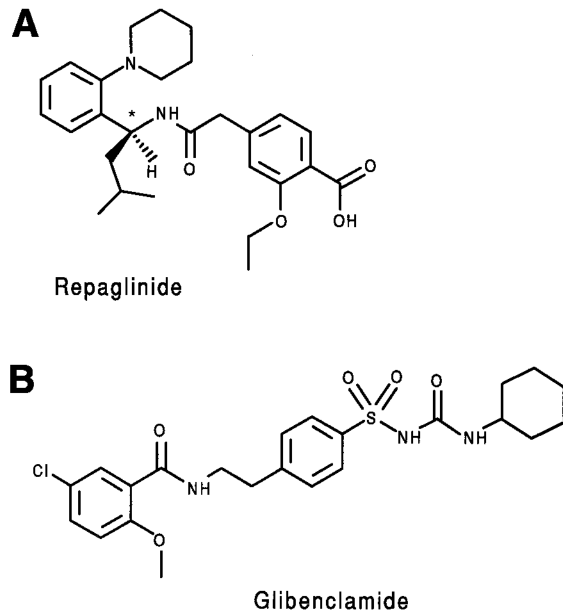
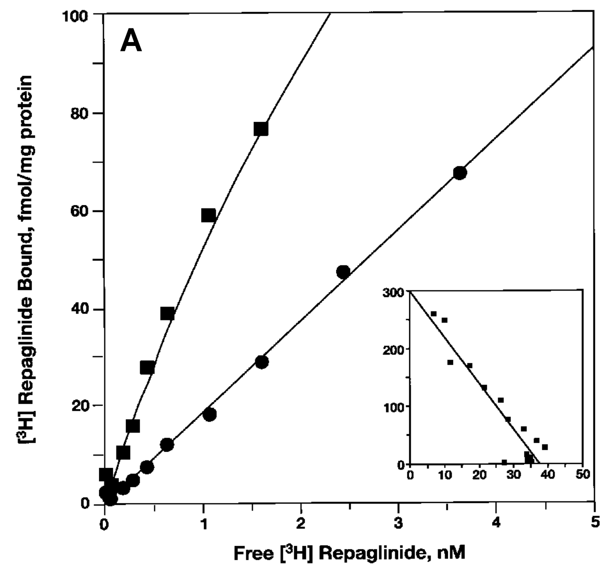


FIG. 1. Chemical structure of repaglinide (*A*) compared with glibenclamide (*B*). *Stereo-specific site of repaglinide.



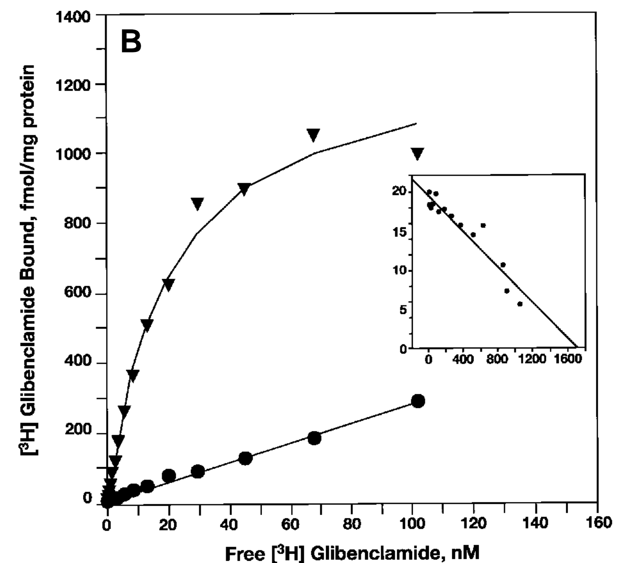


FIG. 2. Data from a single representative saturation experiment in whole βTC3 cells using [^{3}H]repaglinide (*A*) and [^{3}H]glibenclamide (*B*). Binding with repaglinide was saturable; in the range of repaglinide below 3 nmol/l, specific binding (■) exceeded nonspecific binding (●). Binding with glibenclamide was saturable, and specific binding (▼) exceeded nonspecific binding (●). The insets in *A* and *B* illustrate the Scatchard plots of the data; the bound-over-free fraction times 1,000 is plotted against [^{3}H]repaglinide or against [^{3}H]glibenclamide bound in fmol/mg protein.

n = 7) and glibenclamide (K_D 14.4 ± 7.2 nmol/l, *n* = 5). When labeled glibenclamide was used, similar analyses suggested the presence of a single binding site for glibenclamide with a K_D of 24.8 ± 1.4 nmol/l (*n* = 11), whereas two binding sites with K_D values of 8.2 ± 4.2 nmol/l and 550 ± 120 nmol/l (*n* = 4) were detected for repaglinide.

To distinguish pharmacologically the two apparently different binding sites, the binding experiments were also carried out in the presence 10 μmol/l of one of the following drugs: (+)-pentazocin, 1,3-di-(2-tolyl)guanidine, haloperidol, and (+)-3-(3-hydroxyphenyl)-*N*-(1-pro-pyl)-piperidine (PPP). These drugs (in high concentrations) were selected as pharmacological tools purely (if possible) to differentiate the binding sites. Only the latter of the four drugs showed any effect in the assays. In the [^{3}H]glibenclamide assay (Fig. 3*B*), the entire repaglinide curve was shifted to the left (Fig. 4*A*), but the glibenclamide binding was not affected (Fig. 4*B*). Co-incubation with PPP, however, had no effect on the ability of either ligand to displace [^{3}H]repaglinide (data not shown).

Insulin release experiments. The relationship between the secretory response and the concentration of repaglinide and glibenclamide at 5 and 10 mmol/l D-glucose is illustrated in Fig. 4. Repaglinide was more potent at stimulating insulin secretion than was glibenclamide in the presence of either 5 or 10 mmol/l glucose (5 mmol/l glucose at 40 nmol/l, $P < 0.005$; 10 mmol/l glucose at 8 and 40 nmol/l, $P < 0.01$). The relationship between the glucose concentration and the stimulatory actions of repaglinide and glibenclamide was further analyzed in Fig. 5. When applied at roughly equipotent concentrations (40 nmol/l repaglinide and 200 nmol/l glibenclamide; compare with Fig. 4), both compounds shifted the glucose dose-response curve toward the left. Glibenclamide significantly ($P < 0.05$) stimulated insulin secretion at 0 mmol/l glucose, contrary to repaglinide. The maximal stimulatory action was observed at 10 mmol/l D-glucose. As the glucose concentration was elevated further, the relative stimulatory action of both compounds declined. The latter effect was particularly pronounced in the case of repaglinide.

Effects of repaglinide and glibenclamide on exocytosis in voltage-clamped β-cells. It has recently been reported that hypoglycemic SUs, including glibenclamide, possess the capacity to stimulate exocytosis/insulin secretion by a mechanism unrelated to the inhibitory action on the K_{ATP} channels

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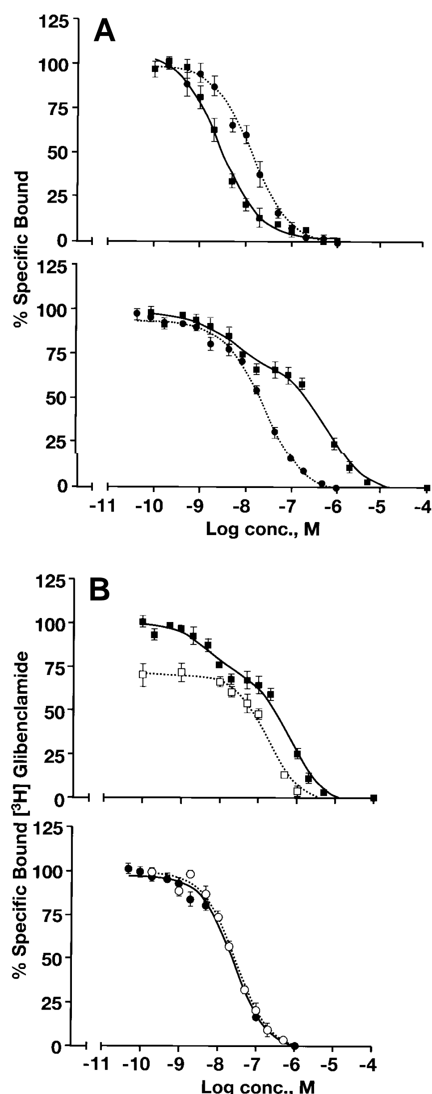

FIG. 3. *A*: Data from competition assays in whole βTC3 cells using 20 pmol/l [^{3}H]repaglinide (upper panel) or [^{3}H]glibenclamide (lower panel). The specific bound radiolabeled ligand (percent) was plotted against the concentration (log scale) of the unlabeled compound added, repaglinide (■) or glibenclamide (●). *B*: Data from competition assays in whole βTC3 cells using 20 pmol/l [^{3}H]glibenclamide. The specific bound radiolabeled ligand (percent) was plotted against the concentration (log scale) of the unlabeled drug added, repaglinide (squares) or glibenclamide (circles) in the absence (solid symbols) or presence of 10 μmol/l PPP (open symbols).

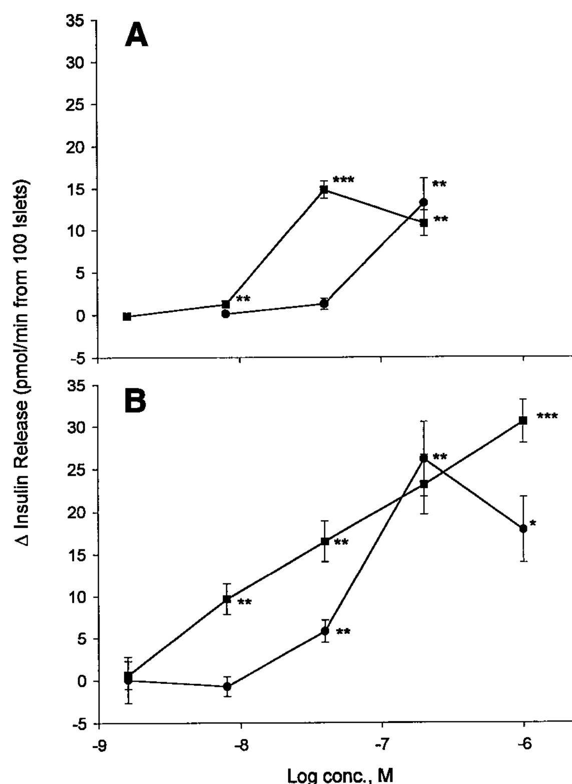

FIG. 4. Dose-dependent stimulation of insulin release by repaglinide (■) and glibenclamide (●) at 5 mmol/l (*A*) and 10 mmol/l (*B*) glucose in perifused mouse islets. Data are mean values ± SE of four to eight experiments (*P < 0.05; **P < 0.01; ***P < 0.001 vs. the reference level [last 10 min before addition of the drugs]).

(10). Here we have examined whether this is also true for repaglinide (Fig. 6). Exocytosis was elicited by depolarizations from –70 to 0 mV. This voltage step activates voltage-dependent Ca^{2+} channels and thus elicits Ca^{2+}-dependent exocytosis. In the absence of repaglinide, the depolarization evoked a capacitance increase of 100 fF (Fig. 6*A*, left). This equates to the discharge of 60 granules, given that each granule contributes 1.7 fF of capacitance upon fusion with the plasma membrane (14). Repaglinide failed to stimulate exocytosis over 6 min when applied at a concentration of 0.1 μmol/l (Fig. 6*A*, right). The same negative results were obtained with a 50-fold higher concentration of the compound (Fig. 6*B*). The effects of 0.1 μmol/l repaglinide are summarized in Fig. 6*C*, which illustrates the amplitude of the exocytotic responses against the time elapsed after addition of the drug. The effect is compared with that of an equal concentration of glibenclamide (10). It can be seen that whereas glibenclamide enhanced exocytosis within 2 min, no such stimulation was obtained with repaglinide. The experiments on repaglinide and glibenclamide were conducted on different sets of cells. It was ascertained that the stimulatory action of glibenclamide on exocytosis and the failure of repaglinide to be effective cannot be accounted for by changes of the integrated Ca^{2+} current (not shown).

In vivo potency of compounds. Fig. 7*A* summarizes the hypoglycemic action of orally administered repaglinide and glibenclamide. The maximum reduction of the blood glucose concentration amounted to ~65% for both drugs. However, the potency of repaglinide was at least one order of magnitude higher than that of glibenclamide. The ED_{50} values were 15.6 μg/kg (95% CI 10.6–22.8 μg/kg) for repaglinide and 203.2 μg/kg (95% CI 143.2–228.4 μg/kg) for glibenclamide (P < 0.001). Following intravenous administration (Fig. 7*B*), the corresponding values were 10.4 μg/kg (95% CI 5.8–18.6 μg/kg) for repaglinide and 70.3 μg/kg (95% CI 35.4–139.6 μg/kg) for glibenclamide (P < 0.001). Again, the maximal hypoglycemic effect was about 65%.

The effects of repaglinide on plasma glucose and insulin levels in mildly diabetic rats are summarized in Table 1. Oral administration of repaglinide produced a strong reduction of

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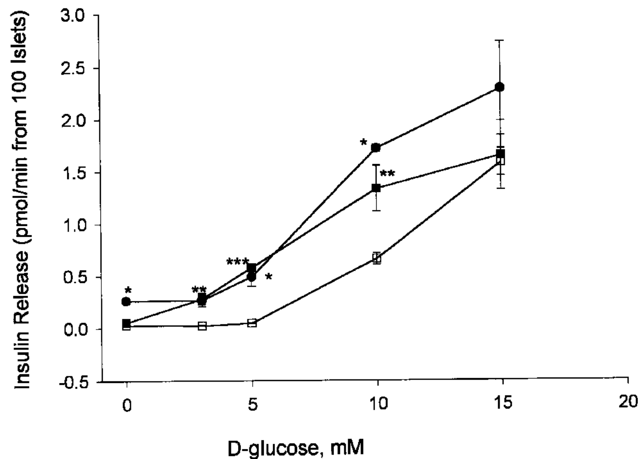

FIG. 5. The influence of D-glucose concentration on the insulin-release response of the drugs at equipotent concentrations of repaglinide (40 nmol/l; ■) and glibenclamide (200 nmol/l; ●) compared with D-glucose alone (□). Data are mean values ± SE of four experiments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

the blood glucose concentration. This was paralleled by a twofold increase in plasma insulin.

DISCUSSION

Repaglinide is a novel orally active insulin secretagogue with insulinotropic action both in vitro and in vivo that lowers the blood glucose concentration in both normal and diabetic rats. We demonstrate that repaglinide and glibenclamide are equally effective in hypoglycemic action but that repaglinide is about 10-fold more potent than glibenclamide, irrespective of whether the drugs were applied orally or intravenously. This suggests that repaglinide represents a potentially useful compound for oral therapy of human type 2 diabetes. Here we have compared the cellular actions of repaglinide with those of glibenclamide. Such studies may be illuminating with regard to the mechanisms behind physiological and pharmacological regulation of insulin secretion in general.

Repaglinide has previously been shown to increase the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) in βTC3-cells, to inhibit the activity of K_{ATP} channels in neonatal rat islet cells (15), and to stimulate insulin secretion in rats islets (16). We have now extended the characterization of the action of repaglinide and compared its in vivo and in vitro effects with those of glibenclamide on receptor binding, insulinotropic capacity, and ability to stimulate insulin release by direct interaction with the exocytotic machinery. In the following discussion, we highlight major differences between the properties of repaglinide and glibenclamide.

Repaglinide and glibenclamide bind to distinct sites. The binding data are easiest to reconcile with the explanation that there are distinct sites for glibenclamide and repaglinide. The observation that the binding of the two drugs is interdependent (i.e., the binding by one drug can be displaced by the other) suggests that the receptors are similar and that the biological actions of the compounds involve common cellular mechanisms. Thus, the stimulation of insulin secretion by repaglinide could be accounted for by the inhibition of the K_{ATP} channel. The resultant reduction of the K^+ permeability produces membrane depolarization, opening of Ca^{2+} channels, elevation of $[Ca^{2+}]_i$, and eventually initiation of Ca^{2+}-dependent exocytosis of the insulin-containing granules. Such an explanation is entirely compatible with the observation that repaglinide blocks cloned K_{ATP} channels (F.M. Ashcroft, personal communication).

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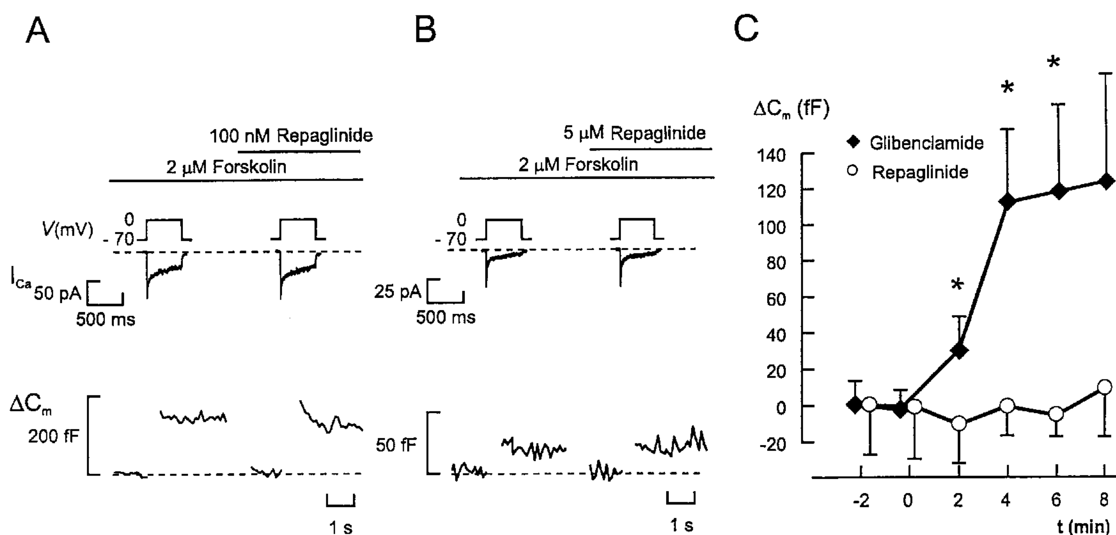

FIG. 6. Failure of repaglinide to stimulate exocytosis in voltage-clamped β-cells. *A* and *B*: Ca^{2+} currents (middle) and increases in cell capacitance (lower) evoked by 500 ms depolarization from –70 to 0 mV (top) before and 4 min after addition of 0.1 µmol/l (*A*) or 5 µmol/l (*B*) repaglinide in the continuous presence of forskolin. *C*: Time-dependent changes in exocytotic responses after addition of 0.1 µmol/l of repaglinide (○) or glibenclamide (◆). There were no systematic changes in the peak Ca^{2+} current. The prestimulatory exocytotic levels (measured at time zero) amounted to 81 ± 11 fF and 54 ± 11 fF in the experiment involving application of repaglinide and glibenclamide, respectively. Data are mean values ± SE of five or six experiments (*$P < 0.05$). The integrated Ca^{2+} currents amounted to 11.8 ± 2.7 and 12.2 ± 3.4 pC ($n = 6$, not different) before and 6 min after addition of 0.1 µmol/l repaglinide. In the four cells where it was possible to record the Ca^{2+} current before and after addition of glibenclamide, it amounted to 4.8 ± 2.2 pC under control conditions and 5.3 ± 3.6 pC 6 min after addition of the sulfonylurea.

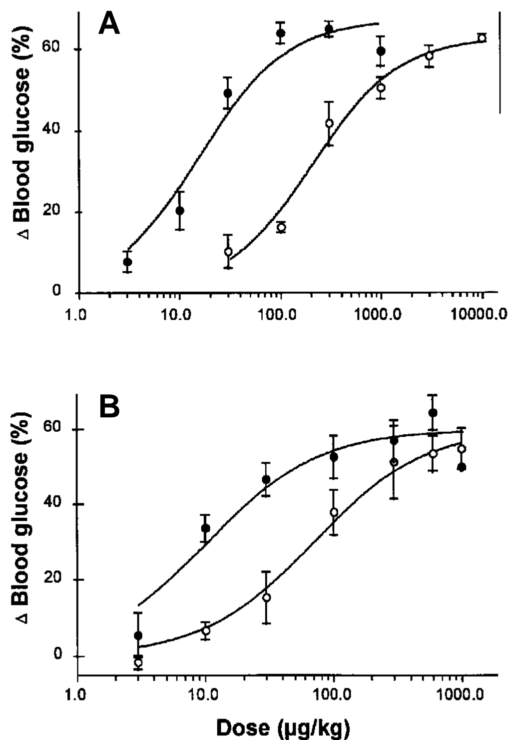

FIG. 7. ***A*****: Antihyperglycemic potencies and efficacies of repaglinide (0.003–1.0 mg/kg; ●) and glibenclamide (0.03–10.0 mg/kg; ○) after oral administration. Data represent the percentage reduction of blood glucose concentration and are mean values ± SE of six determinations.** ***B*****: Same as in** ***A*** **except that repaglinide (0.003–1.0 mg/kg; ●) and glibenclamide (0.003–1.0 mg/kg; ○) were administered as a single intravenous bolus injection. Data are mean values ± SE of four to seven experiments.**

TABLE 1
Effects of repaglinide and vehicle on plasma glucose and plasma insulin levels in mildly diabetic rats

Group	Plasma glucose (mmol/l) 0 min	Plasma glucose (mmol/l) 60 min	Plasma insulin (pmol/l) 0 min	Plasma insulin (pmol/l) 60 min
Vehicle	7.2 ± 0.2	6.2 ± 0.2	159 ± 24	157 ± 19
Repaglinide	6.8 ± 0.2	3.9 ± 0.3*	117 ± 19	248 ± 64†

Data are means ± SE of six experiments. The rats were treated orally with either vehicle or repaglinide, and plasma glucose and plasma insulin levels were measured. *P < 0.001 vs. vehicle group; †P < 0.01 vs. value taken immediately before (0 min) administration of repaglinide (1 mg/kg).

The glibenclamide binding sites present on plasma membranes of βTC3-cells were also examined. The binding parameters for these sites (data not shown) were in agreement with the literature (17) but could not reflect the in vivo and in vitro rank order of potency of the two compounds.

Based on the binding experiments presented here, however, we propose that insulin-secreting cells are equipped with at least two, and possibly three, distinct binding sites for glibenclamide and repaglinide: one with high affinity for repaglinide (K_D < 4 nmol/l) and lower affinity for glibenclamide (14 nmol/l); a second with a high affinity for glibenclamide (25 nmol/l) but low affinity for repaglinide (>500 nmol/l); and a third PPP-sensitive high-affinity (8 nmol/l) repaglinide and glibenclamide site. The sensitivity of two binding sites, but not the third site, to PPP supports the notion that multiple sites for binding exist. The functional significance of the differential PPP sensitivity remains unknown.

Differences in insulinotropic activity. We demonstrate here that repaglinide is five times more potent than glibenclamide in stimulating insulin secretion: half-maximal stimulation is observed at 40 and 200 nmol/l, respectively. This rank order of potency corresponds to the hypoglycemic action in vivo. It is worth noting that glibenclamide, but not repaglinide, stimulated insulin secretion in vitro even in the complete absence of glucose. A further difference was that whereas glibenclamide tended to stimulate insulin secretion even at glucose concentrations as high as 15 mmol/l, the action of repaglinide was confined to intermediate concentrations (Fig. 5). In this context, it is of interest that repaglinide failed to mimic the ability of glibenclamide to stimulate Ca^{2+}-dependent exocytosis by a late mechanism exerted independently of the inhibitory action on K_{ATP} channels (10). Given that repaglinide and glibenclamide are equally potent as K_{ATP}-channel blockers (15), it is tempting to ascribe the stronger stimulatory actions of glibenclamide on insulin secretion at 10 and 15 mmol/l glucose to its capacity to enhance secretion by a direct interaction with insulin exocytosis.

The finding that the action of repaglinide on insulin secretion is particularly pronounced at intermediate (3–10 mmol/l) glucose levels is likely to reflect the synergistic effects of the sugar and the drug on K_{ATP}-channel activity. At low glucose concentrations (0–3 mmol/l), the K_{ATP}-channel activity is high, the membrane potential of the cell is repolarized, and the β-cell is electrically inactive. The addition of repaglinide under these experimental conditions results in the closure of the K_{ATP} channel and the induction of electrical activity. At glucose concentrations that are themselves maximally stimulatory, addition of repaglinide will produce less stimulation, as the K_{ATP} channels are already almost completely inhibited and any additional stimulation must be exerted via more distal mechanisms.

Intracellular actions of SUs and repaglinide. The identity of the granular SUR that mediates the effects on exocytosis is not known. However, the present observation that repaglinide, unlike glibenclamide, does not stimulate exocytosis while being a potent blocker of the K_{ATP} channel clearly distinguishes the mechanism of action of the two compounds. A trivial explanation is differential cellular penetration of the compounds. Whereas glibenclamide is known to be internalized in pancreatic islets (18,19), preliminary data suggest that the intracellular uptake of repaglinide is very limited (W.J. Malaisse, personal communication). It should be pointed out that intracellular uptake of the drug is not required for the stimulatory action. For example, tolbutamide is nearly as effective as glibenclamide in stimulating exocytosis (10) and yet displays no intracellular uptake (18). Given the lipophilic nature of repaglinide, it also seems quite unlikely that it should be excluded from the cell interior. The fact that repaglinide was unable to stimulate exocytosis even

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at concentrations as high as 5 µmol/l strongly argues, therefore, that the molecular mechanisms by which insulin secretagogues close the K_{ATP} channels and stimulate exocytosis are distinct.

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