Original Article

Glibenclamide Treatment Recruits β-Cell Subpopulation Into Elevated and Sustained Basal Insulin Synthetic Activity

Zhidong Ling, Qidi Wang, Geert Stangé, Peter In't Veld, and Daniel Pipeleers

Use of sulfonylureas in diabetes treatment is based on their insulin-releasing effect on pancreatic β-cells. Prolonged action is known to degranulate β-cells, but functional consequences have not been examined at the cellular level. This study investigates influences of in vivo (48-h) and in vitro (24-h) glibenclamide treatment on the functional state of the β-cell population. Both conditions decreased cellular insulin content by >50% and caused an elevated basal insulin biosynthetic activity that was maintained for at least 24 h after drug removal. Glibenclamide stimulation of basal insulin synthesis was not achieved after a 2-h exposure; it required a calcium-dependent translational activity and involved an increase in the percent activated β-cells (50% after glibenclamide pretreatment vs. 8% in control cells). The glibenclamide-activated β-cell subpopulation corresponded to the degranulated β-cell subpopulation that was isolated by fluorescence-activated cell sorter on the basis of lower cellular sideward scatter. Glibenclamide pretreatment did not alter cellular rates of glucose oxidation but sensitized β-cells to glucose-induced changes in metabolic redox and insulin synthesis and release. In conclusion, chronic exposure to glibenclamide results in degranulation of a subpopulation of β-cells, which maintain an elevated protein and insulin synthetic activity irrespective of the presence of the drug and of glucose. Our study demonstrates that the in situ β-cell population also exhibits a functional heterogeneity that can vary with drug treatment. Glibenclamide induces degranulated β-cells with a sustained elevated basal activity that might increase the risk for hypoglycemic episodes.
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Previous studies (1–9) led us to propose that the normal pancreatic β-cell population exhibits intercellular differences that are functionally relevant, in particular for generating the normal dose-response curves to glucose. Chronic exposure to high glucose was found to reduce intercellular differences in glucose sensitivity and thus decrease the net effect of an acute glucose stimulation (7,10,11). Since this view is mainly based on in vitro studies, its potential relevance for the in situ endocrine pancreas needs to be addressed in in vivo models. There are a few reports in which differences among β-cells have been described in the intact pancreas, mostly in terms of the intensity of cellular staining for insulin (12,13). After treatment of rats with the insulin secretagogue glibenclamide, the degranulation of β-cells was not homogenous (12,13). This observation might thus disclose in situ differences in cellular responsiveness to the drug and in subsequent functional activity. The present work examines this possibility by investigating the β-cells that are isolated from glibenclamide-treated rats and comparing them with β-cells isolated from normal control rats either without or with a preincubation with glibenclamide. The data indicate that glibenclamide treatment causes a degranulation in a subpopulation of β-cells; this subpopulation was found to exhibit an elevated basal protein synthetic activity even in absence of the drug. Besides illustrating the in vivo existence of a functional heterogeneity in the β-cell population, our findings demonstrate that this drug, which is used for its acute stimulatory effect on insulin release, exerts additional effects after chronic exposure.

RESEARCH DESIGN AND METHODS

Purification and culture of rat β-cells. Adult male Wistar rats (200–300 g body wt, 10 weeks old) were bred according to Belgian regulation on animal welfare. Use of animals for this study was approved by the local ethical committee. Rats received four intraperitoneal injections of glibenclamide (2 mg/kg body wt; ICN Biochemicals) or 0.9% NaCl (control) twice daily on 2 consecutive days. Pancreatic islets were isolated from control and glibenclamide rats and dissociated in calcium-free medium containing trypsin and DNase (14). Single β-cells were purified by fluorescence-activated cell sorting (FACS), using forward scatter, flavin adenine dinucleotide, and flavin mononucleotide autofluorescence as separation parameters (14). This procedure yielded comparable β-cell numbers ($300–350 \times 10^3$ cells/pancreas) at similar purity (90–95%) from control and glibenclamide animals. After purification, cells were analyzed for their content in insulin and insulin secretory granules, their glucose-dependent metabolic redox state (2), and their rates of insulin release, protein/insulin synthesis, and glucose metabolism during 30 min incubation in absence and presence of glucose.

β-Cell preparations from control rats were also analyzed following culture in absence or presence of glibenclamide. Isolated cells were suspended in Ham's F10 medium (Life Technologies, Strathclyde, U.K.) supplemented with 0.5% (wt/vol) BSA (fraction V; Boehringer Mannheim, Mannheim, Germany), 2 mmol/l glutamine, 2 mmol/l calcium, and 6 mmol/l glucose and reaggregated for 2 h before overnight culture (37°C, 95% air/5% CO_2) (15). Thereafter, cell aggregates were cultured for 2 or 24 h with or without glibenclamide (2 μg/ml), with or without verapamil (10 μmol/l, Isoptine; Knoll, Ludwigshafen, Germany), and with or without cycloheximide (0.5 μg/ml, Sigma, St. Louis, MO). By the end of culture, media were collected for insulin assay, and cells washed and analyzed for their insulin content, and rates of insulin release and protein/insulin synthesis during 30-min incubations in the absence and presence of glucose. In one series of experiments, 24-h cultured control and

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FACS, fluorescence-activated cell sorting.

TABLE 1
Effect of in vivo glibenclamide treatment on insulin content of pancreas and of isolated β-cells

	Control	Glibenclamide
Pancreas content (n = 5)		
Protein content (mg/pancreas)	113 ± 11	93 ± 12
DNA content (mg/pancreas)	5 ± 0.3	5 ± 1
Total RNA content (mg/pancreas)	15 ± 1	14 ± 1
Insulin content (μg/pancreas)	121 ± 7	26 ± 2*
Insulin mRNA (% of control)	100	69 ± 6†
Isolated β-cells		
Insulin content (n = 6) (ng/10^3 cells)	46 ± 3	13 ± 1*
Insulin mRNA (n = 2) (% of control)	100	20–30
Morphometry (n = 3)		
Total cell surface area (μm²/cell)	149 ± 4	133 ± 3
Cytoplasmic surface area (μm²/cell)	127 ± 12	111 ± 3
Secretory vesicles (n/10^2μm² cytoplasmic surface area)	205 ± 12	128 ± 11‡

Data are means ± SE for number of animals (pancreas) or number of independent isolations (isolated β-cells). Statistical significance with control was calculated by Student's t test: *$P < 0.001$, †$P < 0.01$, ‡$P < 0.05$.

glibenclamide cells were dissociated into single cells, sorted by FACS according to their side scatter (14), and then analyzed for their insulin content and their rates of protein/insulin synthesis during 30 min in absence or presence of glucose. In another series, the 24-h cultured control and glibenclamide cells were washed and cultured for an additional 24 h without the drug before their functional activities were determined.

Analysis of protein biosynthesis and insulin release. Samples of freshly isolated or cultured β-cells were incubated at 37°C for 30 or 60 min at different glucose concentrations in 200 μl Ham's F10 medium containing 1% BSA and 50 μCi L-[3,5-^{3}H] tyrosine (specific activity 50 Ci/mmol; Amersham, Bucks, U.K.) (1). Tyrosine concentration was 15 μmol/l with 5 μmol/l present as label with final specific activity of 16.7 Ci/mmol. The incubation was stopped by ice-cold medium containing 1 mmol/l unlabeled tyrosine. After centrifugation, the supernatant was collected for insulin assay and the cell pellets washed before processing for autoradiography (1,2) or extraction in acetic acid (2 mol/l containing 0.25% BSA) for measurement of ^{3}H-protein and ^{3}H-proinsulin (1).

The autoradiographic identification of β-cells in active protein synthesis has been previously described (1,2). Single-cell preparations were fixed in 4% (vol/vol) paraformaldehyde and dried on polylysine-coated glass slides. The preparations were then postfixed for 15 min in 4% (vol/vol) paraformaldehyde before exposure for 4 h to an autoradiographic emulsion (L-4; Ilford, Basildon, Essex, U.K.). The film was developed for 7 min in ID-11 (Ilford) at 20°C and then fixed for another 7 min in Hypam (Ilford). A total of 125 cells were analyzed per condition. Autoradiographic silver grains were counted by epipolarization microscopy at a screen magnification of 5,800 using contrast-enhanced video microscopy. Background labeling, determined in parallel experiments without tracer, was less than two grains per cell. Cells with >10 silver grains were considered as biosynthetically activated. Cell aggregates were fixed in 2.5% glutaraldehyde, postfixed in 1% OsO4, and embedded in Spurr's resin before 1-μm sections were made. Sections were etched with sodium methoxide and exposed for 1 day to autoradiographic emulsion.

Analysis of metabolic responsiveness to glucose. Cellular metabolic responses to glucose were examined by measuring rates of glucose utilization and oxidation in the total cell population and by analyzing the metabolic redox state of individual cells using FACS. Rates of glucose utilization and oxidation were measured as described (16). Samples of 5×10^4 cells were incubated for 2 h at 37°C in 100 μl medium containing 10 μCi D-[5-^{3}H] glucose (Amersham) and 5 μCi D-[U-^{14}C]-glucose (Amersham) at different total glucose concentrations. For FACS analysis, samples of 5×10^4 dissociated β-cells were incubated for 15 min at 37°C at different glucose concentrations. They were then analyzed for their light scatter and NAD(P)H autofluorescence, using a FACS (Facstar; Becton Dickinson, Mountain View, CA) equipped with two argon lasers (Spectra Physics, Mountain View, CA) (2).

Analysis of pancreatic protein content, insulin content, and insulin mRNA. Pancreata were extracted and analyzed for their content in protein (Micro BCA protein assay kit; Pierce), insulin (14), and total RNA. RNA was reverse transcribed with the GeneAmp RNA PCR kit using random hexamer primers and Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer, Norwalk, CT) in standard condition. Insulin mRNA was quantified by competitive RT-PCR (17) using a primer pair insulin-F 5′-TGCCCAGGCTTTTGTCAAAC-3′ and insulin-R 5′-TTCAGACCTTGGCACTGGAG-3′. Competitor DNA was constructed using a two-step PCR method: cDNA from purified rat β-cells was first amplified with primer insulin-R and a linker primer (3′ of insulin-F linked to a sequence 5′-GTACCTGGTGTGTGG-3′ that anneals to the sequence of insulin gene 34 bp downstream of insulin-F) and then amplified with the primer pair insulin-F and -R. This competitor DNA (153 bp) contains the same primer-annealing sequences as the target cDNA, but its PCR product was 34 bp shorter than that of target cDNA (187 bp). After the second amplification, PCR products were purified and concentrated (PCR purification kits; Qiagen, Hilden, Germany) and quantified by spectrophotometry at 260 nm (UV-160-A; Shimadzu). Different concentrations of competitor DNA were then added to the PCR mixtures (Perkin-Elmer) containing cDNA samples. PCRs were carried out in a TC 9700 thermocycler (Perkin-Elmer) in 28 cycles with an annealing temperature of 58°C. Ethidium bromide–stained PCR products were separated by electrophoresis in 2% agarose MS gel (Bioehringer, Mannheim, Germany), detected by ultraviolet transillumination, photographed by a Kodak Digital Science DC40 camera (Kodak, Rochester, NY), and quantified by Boimax ID 3.0 Image Analysis software. The ratios of insulin cDNA to competitor DNA PCR products were plotted against the reciprocal molar amount of competitor DNA. In parallel, a cDNA aliquot was used to amplify the housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (5′-TCCCTCAAGATTGTCAGCAA-3′ and 5′-AGATCCACAACGGATACATT-3′) to confirm equal mRNA loading.

Data expression. Data were calculated as function of the number of β-cells counted on day 1 or after dissociation of cultured aggregates. Results represent means ± SE. Statistical significance of differences was calculated by ANOVA or Student's t test.

RESULTS

Insulin content and biosynthetic activity of β-cells from glibenclamide-treated rats. When adult rats were treated for 2 days with glibenclamide, their pancreatic insulin content decreased by 80% and insulin mRNA content by 30%, whereas the corresponding total protein and RNA content remained unchanged (Table 1). β-Cells

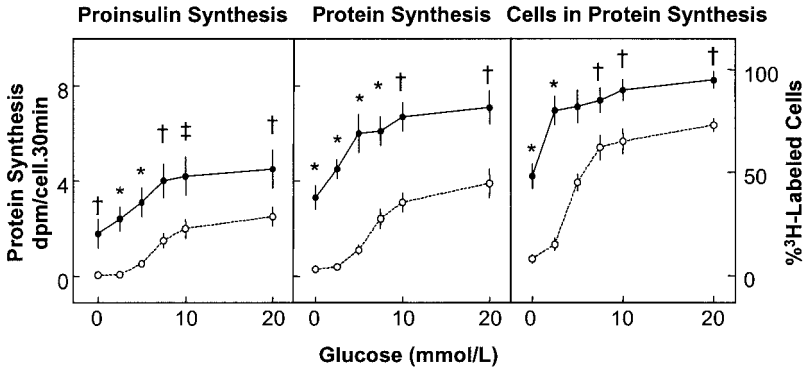

FIG. 1. Effect of glibenclamide pretreatment in vivo on protein synthetic activity by isolated rat β-cells. Cells isolated from glibenclamide-treated (●) and control (○) animals were incubated with ^{3}H-tyrosine for 30 min at different glucose concentrations and analyzed for the rates of insulin synthesis and total protein synthesis as well as for the percent of biosynthetically activated cells in autoradiographs. Data represent the means ± SE of three to five independent experiments. Statistical significance of differences between control and glibenclamide cells was calculated by Student's t test: *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$.

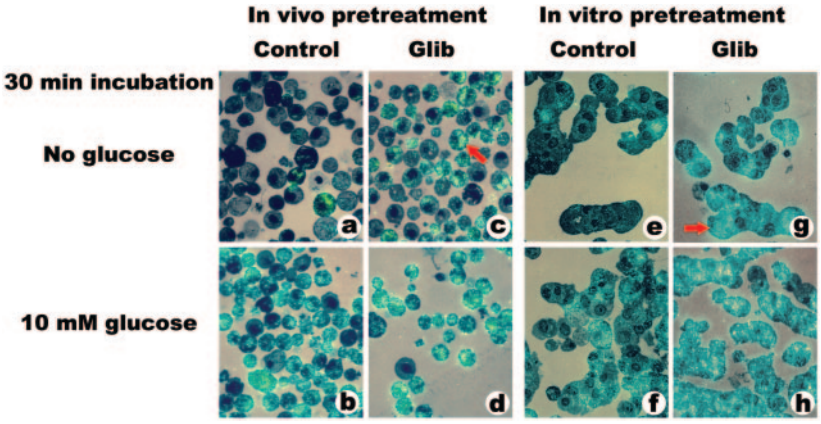

FIG. 2. Effects of glibenclamide (Glib) pretreatment in vivo (*A–D*) and in vitro (*E–H*) on the degree of ^{3}H-tyrosine incorporation by isolated rat β-cells. After glibenclamide pretreatment, β-cells were incubated for 30 min with ^{3}H-tyrosine in absence (*A,C,E,G*) or presence (*B,D,F,H*) of 10 mmol/l glucose. Autoradiographs of the cells are shown in epipolarization microscopy. In absence of glucose, silver grains (green-yellow dots) accumulated in the majority of glibenclamide-treated cells (arrow) but not in control cells; at 10 mmol/l glucose, virtually all glibenclamide cells became active as well as the majority of control cells. Unlabeled cells are gray; nuclei are black.

isolated from glibenclamide rats (glibenclamide cells) contained 70% less insulin mRNA and peptide than β-cells from control rats (Table 1). Their degranulated state was also documented by the 40% lower number of secretory vesicles per cytoplasmic surface area ($P < 0.05$, Table 1).

When purified β-cells were incubated for 30 min, glibenclamide cells exhibited higher rates of proinsulin synthesis irrespective of the presence or concentration of glucose (Fig. 1). In the absence of glucose, their insulin synthetic activity was already at the level of that in control cells stimulated by 10 mmol/l glucose (Fig. 1). The net glucose-induced increase was comparable in amplitude with that in control cells, but the half-maximal effect was reached at 5 mmol/l vs. 7 mmol/l glucose in control cells. At maximal glucose stimulation, the rates of insulin synthesis were twofold higher than in control cells (Fig. 1). Similar differences were noticed in total protein synthesis (Fig. 1).

These higher rates of insulin and protein synthesis are mainly the result of a higher percent of β-cells that are biosynthetically active. Autoradiographs of ^{3}H-tyrosine–labeled cells visualized the percent cells that contributed to the measured rates of protein synthesis (Figs. 1 and 2). After in vivo glibenclamide treatment, almost 50% of isolated β-cells were biosynthetically active when incubated in the absence of glucose vs. <10% of control cells ($P < 0.05$, Figs. 1 and 2). Glucose dose-dependently increased the percentage of biosynthetically active cells in both preparations, with half-maximal effects at 1.6 (glibenclamide) and 4.5 (control) mmol/l. At maximal glucose stimulation (20 mmol/l), 95% of glibenclamide-pretreated cells were activated vs. 73% in control cells ($P < 0.01$, Fig. 1).

Metabolic state and responsiveness of β-cells from glibenclamide-treated rats. Over a 2-h incubation, the rates of glucose utilization and oxidation by glibenclamide cells were almost identical to those in control cells when measured in absence or at low glucose, while slightly, but not significantly higher at 7.5–20 mmol/l glucose (Fig. 3). When individual cell responses to glucose were analyzed through the glucose-induced increase in cellular NAD(P)H fluorescence, the percent cells responding to glucose was similar in both preparations. However, the glibenclamide population contained a higher percent cells (27 ± 3 vs. 14 ± 2% in control) with elevated NAD(P)H content in absence of glucose: their fluorescence intensity was comparable with that of control cells after glucose stimulation (Fig. 4).

Functional activity of β-cells following glibenclamide treatment in vitro. To assess whether the changes in β-cells isolated from glibenclamide rats resulted from direct effects of the drug, we repeated the above analysis on β-cells isolated from normal control rats and incubated for 2 or 24 h with glibenclamide.

During 24-h culture with glibenclamide, β-cells released threefold more insulin than control cells cultured without the drug (Table 2). At the end of culture, their insulin content was 44 ± 2% lower (Table 2) and their insulin mRNA content 40 ± 11% lower ($n = 3$). When the cells were then tested for their biosynthetic activity over a 30-min incubation following washout of the drug, their rates of protein and insulin synthesis were significantly higher than those in control cells (Fig. 5). Glibenclamide cells were also more sensitive to the glucose-induced stimulation of protein/proinsulin synthesis, but maximal rates were comparable with those in control cells. Autoradiographs indicated that a higher percentage of glibenclamide cells were biosynthetically active in absence of glucose (>50 vs. 10% in control) and that glucose increased the percent activated cells in both preparations (Fig. 2). These signs of increased biosynthetic activity in glibenclamide-pretreated cells were maintained during a subsequent 24-h culture period without the drug: when the cells were analyzed at the end of this glibenclamide-free period, they exhibited dose-response curves that were similar to those measured immediately after glibenclamide culture (Fig. 5). Glibenclamide-pretreated cells also exhibited a higher secretory activity (Fig. 5), and this was maintained during the subsequent 24-h glibenclamide-free

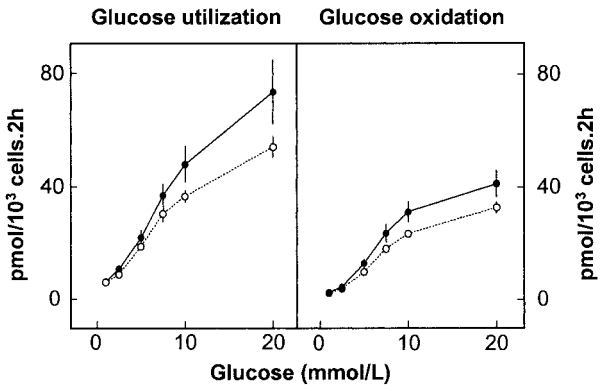

FIG. 3. Glucose utilization and oxidation by β-cells isolated from control (○) and glibenclamide-treated (●) rats. Data represent the means ± SE of four independent experiments.

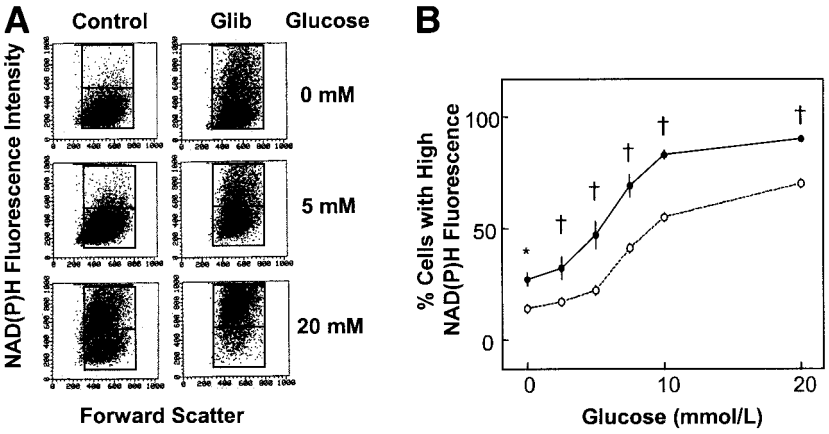

FIG. 4. Dot plots after FACS analysis of β-cells that were isolated from glibenclamide-treated and control rats and then incubated for 15 min at different glucose concentrations. *A*: Windows were set to delineate the β-cell subpopulations with high and low cellular NAD(P)H autofluorescence intensity. *B*: The percent cells detected in these windows are plotted as means ± SE of 9 or 10 independent experiments. Statistical significance of differences between control cells (○) and glibenclamide cells (●) was calculated by Student's *t* test: †$P < 0.01$; *$P < 0.001$.

period (4 ± 1 ng/10^3 cells vs. 1.7 ± 0.3 ng/10^3 control cells, $P < 0.05$). Glucose dose-response curves were shifted to the left, indicating a higher sensitivity to glucose stimulation.

When glibenclamide pretreatment was only for 2 h, the cells exhibited an elevated insulin release during a 30-min incubation period but not an elevated protein synthetic activity (Fig. 5).

Heterogeneity in glibenclamide-induced degranulation and correlation with subsequent functional activity. β-Cells isolated from control rats exhibit intercellular differences in insulin content. When the cells are distributed according to their density in secretory vesicles (SV; number SV/$10^2\mu m^2$), >85% of the cells contained >150 SV/$10^2\mu m^2$ (categorized as normal 218 ± 49/10^2 μm^2), 10% between 75 and 150 SV/$10^2\mu m^2$ (mild degranulation), and <5% <75 SV/$10^2\mu m^2$ (severe degranulation) (Fig. 6*A*). After in vivo glibenclamide treatment, the percent of mildly and severely degranulated cells increased, but 30% of the cells still ranked as normal (198 ± 39/10^2 μm^2) (Fig. 6*A*). In vitro glibenclamide treatment also resulted in a heterogeneously distributed degranulation (Fig. 6*B*). It is to be noticed that the 48-h culture period caused in itself some degree of degranulation in control cells: 172 ± 52 vs. 204 ± 59 SV/$10^2\mu m^2$ immediately after isolation ($P < 0.001$).

In view of the intercellular differences in glibenclamide-induced degranulation, we examined whether the cells with more pronounced degranulation corresponded to those with increased protein synthetic activity. FACS was used to isolate a β-cell subpopulation with lower side scatter, a parameter that is known to decrease with the granule density of cells (Fig. 7). As expected, the 24-h glibenclamide preparation yielded more cells in the low side scatter window than the control preparation (Fig. 7). This glibenclamide low side scatter subpopulation contained cells with a 50% lower mean insulin content than the glibenclamide high side scatter; its mean insulin content was 65% lower than that in the control high side scatter subpopulation where the majority of control β-cells were recovered (Table 3). When comparing protein/proinsulin synthetic activities of cell subpopulations sorted according to their degree of granulation, it was found that the severely degranulated glibenclamide low side scatter cells exhibited twofold higher biosynthetic rates at 2.5 mmol/l glucose than the glibenclamide high side scatter or control cells with a higher cellular insulin content (Table 3). These data indicate that the higher basal biosynthetic activities after glibenclamide treatment are achieved by the subpopulation of cells that became severely degranulated by the drug treatment.

Role of calcium and protein translation in glibenclamide-induced recruitment of β-cells into high basal activity. The correlation between cellular degranulation and increased basal protein synthetic activity raises the question whether interference with degranulation would also interfere with the elevation in biosynthetic rates. Glibenclamide pretreatment was therefore conducted at low calcium levels or in the presence of a calcium uptake inhibitor, conditions that are known to interfere with the insulin-releasing effect of glibenclamide and hence cellular degranulation (Table 2).

The glibenclamide-induced elevation in basal protein

TABLE 2
Effect of verapamil on glibenclamide-induced stimulation of rat β-cell functions

Cell preparations		Insulin release during culture (ng/10^3 cells/24 h)	Insulin content (ng/10^3 cells)	Total protein synthesis		Insulin synthesis	
				0 mmol/l glucose (10^3 dpm/10^3 cells/30 min)	10 mmol/l glucose (10^3 dpm/10^3 cells/30 min)	0 mmol/l glucose (10^3 dpm/10^3 cells/30 min)	10 mmol/l glucose (10^3 dpm/10^3 cells/30 min)
Glib	Verapamil						
−	−	3.8 ± 0.9	31.2 ± 2.5	0.7 ± 0.1	4.7 ± 0.4	0.3 ± 0.04	3.3 ± 0.03
+	−	13.5 ± 1.8*	17.2 ± 1.0*	3.9 ± 0.6*	6.9 ± 0.6†	2.1 ± 0.2*	3.6 ± 0.2
−	+	3.2 ± 0.7	30.4 ± 3.0	0.8 ± 0.03	4.4 ± 0.5	0.4 ± 0.1	2.7 ± 0.2
+	+	4.0 ± 0.7	31.9 ± 4.4	1.0 ± 0.1	4.3 ± 0.4	0.4 ± 0.6	2.6 ± 0.1

Data are means ± SE of five independent experiments. β-Cells were cultured for 24 h without or with glibenclamide in the absence or presence of verapamil. At the end of this culture period, medium was collected for measuring insulin release and cells washed and incubated in the absence of glibenclamide to measure rates of protein and insulin synthesis during a 30-min incubation. Statistical significance of differences was calculated by ANOVA. Differences between conditions with and without glibenclamide either in the presence or absence of verapamil: *$P < 0.01$, †$P < 0.001$.

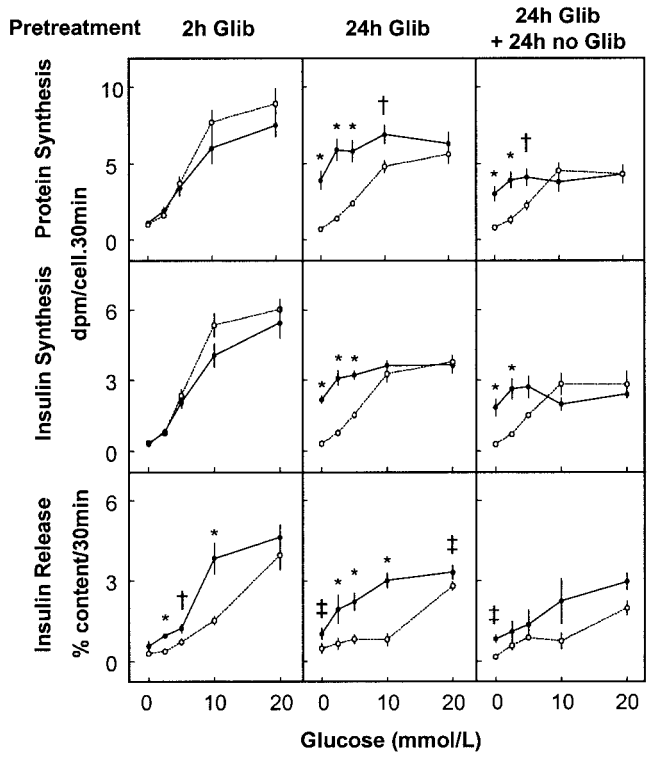

FIG. 5. Effect of glibenclamide pretreatment in vitro on glucose-induced protein synthesis, insulin synthesis, and insulin release by rat β-cells. β-Cell preparations from control rats were precultured for 2 or 24 h with (●) or without (○) glibenclamide, washed, and cultured for another 24 h in control medium. Data represent the means ± SE of four to six independent experiments. Statistical significance of differences between control and glibenclamide-pretreated cells was calculated by Student's *t* test: †$P < 0.01$; *$P < 0.001$; ‡$P < 0.05$.

synthetic activity was not observed when the cells were cultured at 0.3 instead of 2 mmol/l calcium (data not shown) or when the calcium channel blocker verapamil was added to the 2 mmol/l calcium medium (Table 2). It is unlikely that the calcium-dependent increase in extracellular insulin concentration was responsible for the increase in basal protein biosynthetic activity, since the latter was not increased when control β-cells were precultured in presence of insulin (2.10^{-7} mol/l): protein synthesis at 0 mmol/l glucose: 0.7 ± 0.1 dpm/cell in control preparations and 0.8 ± 0.2 in insulin-treated cells and at 10 mmol/l glucose: 4.9 ± 0.7 in control cells and 5.6 ± 0.2 in insulin-treated cells ($n = 4$). It is thus conceivable that prolonged glibenclamide-induced changes in intracellular calcium handling influence protein synthesis regulation leading to an increase in basal protein synthetic activity. Such a mechanism is supported by the observation that

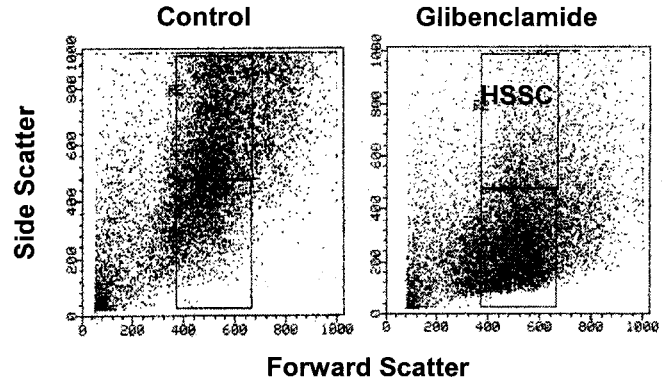

FIG. 7. Sorting of degranulated β-cell subpopulation following glibenclamide pretreatment in vitro. Figure shows dot blots for cellular sidewards scatter and forward scatter intensities during FACS analysis of β-cells after 24-h culture with or without glibenclamide and subsequent dissociation. After glibenclamide treatment, the majority of β-cells were recovered as a subpopulation with lower side scatter intensity than control cells, while their forward scatter intensities were comparable. Windows delineate the subpopulations that were separated and found to be degranulated (low side scatter, LSSC) or normally granulated (high side scatter, HSSC).

the glibenclamide-induced activation of basal protein synthesis did not occur when glibenclamide pretreatment occurred in the presence of cycloheximide, an inhibitor of protein translation (Table 4). When added to the 24-h culture medium, cycloheximide reduced protein synthesis by 67% (3.6 vs. 11.0 ± 1.0 dpm/cell in controls, $n = 3$, $P < 0.05$).

DISCUSSION

Glibenclamide belongs to the second generation sulfonylureas that have been used for decades in the treatment of type 2 diabetes. The agents stimulate insulin release through mechanisms that have been extensively investigated in isolated pancreas and islet preparations (18–21). Their binding to a surface membrane receptor induces closure of an associated ATP-sensitive K^+ channel that leads to membrane depolarization, opening of voltage-dependent Ca^{2+} channels, and rise in cytoplasmic free calcium concentration to levels that activate exocytosis (19). At variance with first-generation sulfonylureas, the compounds also generate a secretory activity after their removal from the extracellular medium (22). This sustained effect coincides with cellular accumulation of the drug, primarily in membranes of secretory vesicles and mitochondria (22–25). It has been attributed to a protein kinase C activation following glibenclamide stimulation of diacylglycerol synthesis (21,26). During these in vitro studies up to 2-h incubation periods, the sulfonylurea

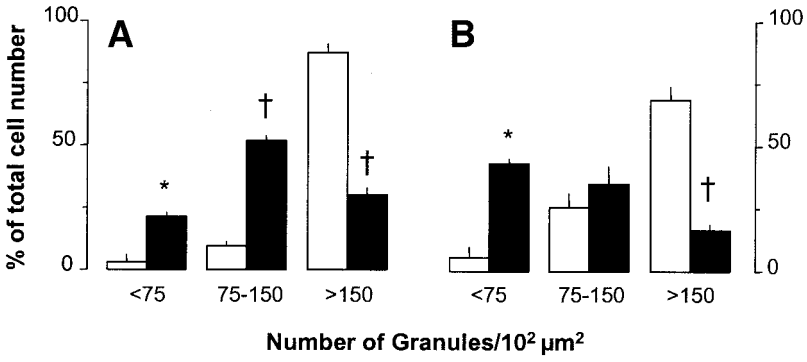

FIG. 6. Heterogeneity in glibenclamide-induced degranulation of β-cells. Cells were distributed according to their number of secretory granules. Comparison of β-cell preparations without (□) or with (■) glibenclamide pretreatment either for 2 days in vivo (*A*) or for 24 h in vitro (*B*). Data represent percent cells with the indicated range in number of secretory granules and are expressed as the mean ± SE of three independent experiments (30–35 cells analyzed per experiment). Statistical significance of differences between control and drug-treated preparations was calculated by Student's *t* test: *$P < 0.01$; †$P < 0.001$.

TABLE 3
Characteristics of glibenclamide-induced β-cell subpopulation

Subpopulations	Cell number (% total)	Insulin content (ng/10^3 cells)	Protein synthesis (10^3 dpm/10^3 cells/h)	Insulin synthesis (10^3 dpm/10^3 cells/h)
Control				
High side scatter	57 ± 1	41 ± 6	4.4 ± 0.5	4.8 ± 1.4
Low side scatter	43 ± 1	25 ± 3	6.1 ± 1.8	5.4 ± 2.0
Glibenclamide				
High side scatter	29 ± 2	29 ± 4	7.3 ± 0.6	5.8 ± 1.6
Low side scatter	71 ± 2	15 ± 3	14.5 ± 1.8*†	11.6 ± 2.2

Data are means ± SE of four independent experiments. β-Cells cultured for 24 h with or without glibenclamide were dissociated into single cells and separated into subpopulations with high or low side scatter. The isolated subpopulations were analyzed for cell number, cellular insulin content, and rates of protein and insulin synthesis during 1-h incubation at 2.5 mmol/l glucose without glibenclamide. Statistical significance of differences was calculated by ANOVA. Differences between values in low and high subpopulations: *$P < 0.05$; between glibenclamide and control in the high or low populations: †$P < 0.001$.

stimulation of insulin release was not associated with an increase in insulin synthesis (27–30). When administered to normal rodents, the drugs induce elevated basal insulin levels and result in degranulation of β-cells (31). In the present study, rats treated with glibenclamide presented a 70% reduction in pancreatic insulin reserve. Their degranulated β-cells were purified by flow cytometry and were found to exhibit elevated basal rates of insulin synthesis and release compared with β-cells in control rats. These characteristics were reproduced in vitro by preculturing β-cells for 24 h in the presence of glibenclamide, indicating that they result from a direct drug interaction with the β-cells. Both in vivo and in vitro modes of causing degranulation and elevated basal activities were found to have induced a sustained alteration in the functional state of the β-cells, as these properties were detected in the absence of glucose and maintained for at least 24 h in the absence of the drug. Our data also provide evidence that β-cells that degranulated following glibenclamide treatment exhibit a sustained elevation of insulin synthetic and secretory activities under basal conditions. These cells maintain elevated insulin levels at low glucose concentrations and may thus be the cause for hypoglycemic episodes in drug-treated patients.

Glibenclamide treatment did not degranulate all β-cells, as was also noticed in previous studies (12,13). When isolated, 30% of the β-cells from drug-treated animals had the same density in secretory vesicles and the same insulin content as the majority (85%) of β-cells from control animals. This observation, together with the detection of an altered functional state in degranulated β-cells, demonstrates that the in situ pancreatic β-cell population also exhibits intercellular differences and thus presents a functional heterogeneity. Since the currently described glibenclamide effects are calcium dependent, it is conceivable that they reflect intercellular differences in calcium metabolism (32).

Autoradiographs showed that prolonged exposure to glibenclamide recruited β-cells into a state with elevated protein synthetic activity under basal conditions. Without prior drug treatment, only 8% of the β-cells were scored as biosynthetically active when incubated in absence of glucose, whereas this was 50% after glibenclamide treatment. Both percentages dose-dependently increased in the presence of glucose, illustrating that glibenclamide-pretreated cells remained sensitive to the glucose-induced recruitment that is known to occur in the normal β-cell population (1,2). In all test conditions, the percent activated cells was significantly higher in the glibenclamide-pretreated population, which accounts for its four- to sevenfold higher basal rates and twofold higher maximal rates of insulin synthesis. The amplitude of the glucose-inducible effect was similar in both preparations but was reached at a lower concentration in glibenclamide-pretreated β-cells. This resulted from a higher effectiveness of the glucose-induced signal rather than from a higher rate of glucose metabolism. Glibenclamide pretreatment did not cause changes in the rates of glucose utilization and oxidation, as was also the case during shorter incubations with the drug (22). It did not alter the number of β-cells that exhibited a rapid metabolic redox response to glucose.

The glibenclamide-induced activation of β-cells did not occur when translation was blocked by cycloheximide or when no rise in intracellular calcium could occur such as

TABLE 4
Effects of cycloheximide on glibenclamide-induced activation of β-cells

Subpopulations		Insulin release during culture (ng/10^3 cells/24 h)	Insulin content (ng/10^3 cells)	Protein synthesis	
				0 mmol/l glucose (10^3 dpm/10^3 cells/h)	10 mmol/l glucose (10^3 dpm/10^3 cells/h)
Glibenclamide	Cyclohexmide				
−	−	8.9 ± 2.5	22.2 ± 1.1	1.2 ± 0.2	11.1 ± 1.6
+	−	20.4 ± 2.6*	12.8 ± 3.0	5.3 ± 0.6†	11.2 ± 0.6
−	+	10.8 ± 0.5	19.2 ± 1.5	1.4 ± 0.3	7.7 ± 0.6
−	+	15.0 ± 2.2	13.2 ± 2.2	1.5 ± 0.2‡	6.1 ± 0.7§

Data are means ± SE of three or four independent experiments. β-Cells were cultured for 24 h without or with glibenclamide in the absence or presence cyclohexmide. At end of culture, cells were washed and their protein synthesis was measured during 1-h incubation without glibenclamide and cyclohexmide. Statistical significance of differences was calculated by ANOVA. Differences between conditions with and without glibenclamide either in presence or absence of cyclohexmide: *$P < 0.05$, †$P < 0.001$; between conditions with and without cyclohexmide in either the presence or absence of glibenclamide: ‡$P < 0.001$, §$P < 0.05$.

at low medium calcium concentration (0.3 instead of 2 mmol/l) or in presence of the calcium chelator verapamil. Since these conditions are known to suppress sustained insulin release, it could be hypothesized that the hyperactivated state of the cells is a consequence of their degranulation. Alternatively, a sustained glibenclamide-induced elevation of intracellular calcium (33) resulting from intracellular accumulation of glibenclamide (23) may cause changes in the regulation of protein translation leading to an altered synthetic activity that is best detected in the absence of glucose, the β-cells' main regulator of translation. Such mechanisms could account for the increased rates of insulin synthesis at low glucose; it would also explain why more insulin is synthesized by cells with a lower insulin mRNA expression. Calcium-dependent regulation of translation has been reported in other cell types (34). In β-cells, it may lead to higher expression of calcium-regulated proteins such as functional Ca^{2+} channels (35).

In conclusion, prolonged exposure of rat β-cells to the insulin secretagogue glibenclamide results in degranulation of a subpopulation of cells and their activation into elevated protein and insulin synthesis, a state that is maintained in absence of the drug and of glucose. This glibenclamide-induced recruitment of β-cells into an altered functional state is calcium dependent and involves translational activity. This effect does not interfere with or alter the cellular rates of glucose oxidation but makes the cells more sensitive to glucose-induced changes in metabolic redox, insulin synthesis, and release. Our data demonstrate that the pancreatic β-cell population in vivo also exhibits a functional heterogeneity and that degranulated β-cells during sulfonylurea treatment correspond to cells with an elevated basal activity.

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