Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 4372–4377, April 1997
Biochemistry

Modulation of glucose responsiveness of insulinoma β-cells by graded overexpression of glucokinase

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Communicated by Shirley M. Tilghman, Princeton University, Princeton, NJ, February 13, 1997 (received for review November 25, 1996)

ABSTRACT **Insulinoma β-cells capable of overexpressing glucokinase under the control of a doxycycline-dependent transcriptional transactivator were established from parental INS-1 cells. Glucokinase could be maximally induced to a level more than 20 times the basal level after 36 h of culture with doxycycline. Intermediate levels of induction could be achieved by varying doses of, and time of culture with, the inducer. The rate of glycolysis was measured in cells with 3-, 5-, and 8-fold increment in glucokinase activity above the noninduced level. Proportionate increases in glycolytic flux occurred in cells cultured at low physiological glucose concentration. At high glucose concentration, induction of glucokinase in excess of 2-fold above basal resulted in little additional increase in glycolysis. The consequences of graded increases of glucokinase on two physiological glucose effects were investigated. Increments in glucokinase activity were accompanied by a stepwise shift to the left of the dose–response curve for the inductive effect of glucose on the L-type pyruvate kinase mRNA. Similarly, the insulin secretory response to glucose was shifted leftward in glucokinase-induced cells. The following conclusions are drawn: (*i*) glucokinase is the major rate-limiting enzyme for glycolysis in these cells; (*ii*) downstream metabolic steps become limiting at high extracellular glucose concentration with moderate increases in glucokinase over the wild-type level; (*iii*) within limits, glucokinase activity is a determining factor for two types of glucose responses of the β-cell, the induction of specific gene expression, and insulin release.**

The endocrine β-cell of the pancreas contains glucokinase, a hexokinase isoenzyme with distinctive kinetics, and tissue expression shared by only a few cell types in the body, including mostly the liver besides the endocrine pancreas (1–3). Half-saturation of glucokinase with glucose occurs at a substrate concentration around 6 mM, close to the concentration of glucose in the extracellular fluids (4). Because glucose transport in the β-cell is not rate-limiting (5), the glucose concentration in the intracellular space is close to the extracellular concentration, physiologically around 5 mM. Thus, glucokinase operates in the cell under conditions close to half-saturation with glucose, allowing changes in the extracellular glucose concentration to be translated into parallel changes in the rate of glucose phosphorylation, the first step of glucose metabolism. In addition, glucose phosphorylation appears to be the major rate-limiting step in the glycolytic pathway in the β-cell, because assayable glucokinase activity is the lowest of the activities of all glycolytic enzymes, and glucose utilization displays a glucose concentration dependency similar to that of glucokinase (6). Therefore, glucokinase is considered to be the enzyme that allows glucose to regulate its own rate of metabolism in the β-cell.

Important physiological effects, including effects on the rate of specific gene transcription and insulin secretion, are elicited in the endocrine β-cell by changes in the extracellular glucose concentration. These responses reflect a direct, rather than hormone-mediated, effect of glucose at the level of the β-cell. Moreover, they depend on an increase in the cellular metabolism of glucose. Because the rate of glucose metabolism is thought to be largely determined at the glucokinase step, glucokinase is often viewed as the "glucose sensor" for the physiological responses of the β-cell to the sugar (7). Consistent with this view, human subjects with inherited mutations of glucokinase (maturity onset diabetes of the young, MODY 2) present a disorder in their capacity to secrete insulin in response to hyperglycemia (8). Pancreas perfusion experiments in transgenic mice expressing glucokinase anti-sense RNA in the β-cells similarly demonstrated a rightward shift in the dose–response curve of insulin release elicited by glucose (9). Conversely, islets of transgenic mice with a yeast hexokinase transgene active in β-cells displayed an increase in glucose-dependent insulin secretion (10).

The possibility of manipulating the level of glucokinase activity by gene transfer experiments offers an interesting approach to test the regulatory role of this enzyme in glucose metabolism. An attempt in this direction, using whole rat islets and adenovirus-mediated transduction of the glucokinase gene placed under control of the cytomegalovirus promoter, yielded negative results (11). However, the conclusions from that study are limited, because neither the kinetics nor the level of glucokinase expression in β-cells were controlled, such that only extremely strong overexpression of the enzyme could be studied. Here, we describe the establishment, from the well differentiated parental INS-1 cell line (12), of an insulinoma cell clone capable of expressing glucokinase under the control of the tetracycline-dependent reverse transactivator of Bujard and colleagues (13). This system permitted to set glucokinase activity at precisely defined levels in the cells. Investigations of the effects of incremental degrees of glucokinase induction on the rate of glucose metabolism, glucose-stimulated gene expression, and insulin secretion are reported below.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection. The INS-1 cell line and derived clones were cultured in RPMI medium 1640 with fetal bovine serum, penicillin-streptomycin, Na pyruvate, and mercaptoethanol as described (14). Stable transfection was performed using the Ca-phosphate-DNA coprecipitation method followed by glycerol shock (15). To establish cells expressing the reverse tetracycline-dependent transactivator, INS-1 cells in 10-cm dishes were transfected with 15 μg of plasmid

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0027-8424/97/944372-6$2.00/0
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PUHG17–1 (13), plus 3 μg of RSVNeo (16). After selection with 350 μg/ml G418 for 21 days, resistant colonies were isolated with cloning rings. Individual clones were tested for expression of reverse tetracycline-dependent transactivator by transient transfection of PUHC13–3, a reporter plasmid with the luciferase gene driven by a minimal cytomegalovirus promoter complemented with multiple copies of the tetracycline operator DNA element (13). One clonal line that exhibited very high tetracycline-inducible luciferase activity and undetectable basal luciferase activity, was chosen and used for a second round of transfection with a glucokinase expression plasmid. This plasmid was constructed by inserting a *Eco*RI fragment of plasmid pB-GK2, containing the rat liver glucokinase cDNA (17), downstream of the tetracycline operator-cytomegalovirus minimal promoter in plasmid PUHD10–3 (18). Plasmid Tk-hygromycin (18) was cotransfected for selection by culture in presence of 200 μg/ml hygromycin. Individual resistant colonies were cloned and maintained in long-term culture with 150 μg of G418/ml and 100 μg of hygromycin/ml. Clones were screened for high glucokinase activity after 24 h of cell culture with 1,000 ng of doxycycline/ml.

Assay of Glucokinase. Cells were scraped from 6-cm culture dishes and washed twice in ice-cold PBS. The cells were suspended and allowed to swell for 10 min at 4°C in 150 μl of a hypotonic buffer described previously (19), with addition of 0.13 mM phenylmethylsulfonyl fluoride. Potassium chloride (or sodium chloride for Western blotting) was added to a final concentration of 80 mM, and the cells were lysed by three cycles of freezing-thawing. The lysates were centrifuged in a microfuge at top speed and 4°C for 30 min to obtain a particle-free supernatant. The protein concentration was analyzed by the Bradford assay. Hexokinase activity was measured at 30°C by a glucose-6-phosphate dehydrogenase-coupled assay with fluorometer estimation of NADH production as described previously (19). Glucokinase activity was calculated as the difference in NADH produced at 100 mM and 0.5 mM glucose and expressed in nmol/min (= mU) per mg of protein. Immunoblotting of glucokinase was performed as described (2), except that the sheep antibodies to rat liver glucokinase were detected with peroxidase-conjugated rabbit antibodies to sheep immunoglobulin, using an enhanced chemiluminescence revelation system (Pierce).

Rate of Glucose Utilization. The rate of glycolysis was estimated from the production of [^{3}H]water from D-[5-^{3}H]glucose (20). Cell monolayers in 48-well dishes were washed twice in Krebs–Ringer solution containing 140 mM NaCl, 3.6 mM KCl, 1.5 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.5 mM Na_2HPO_4, 2 mM NaH_2CO_3, 10 mM Hepes (pH 7.4), and 0.07% BSA and incubated at 37°C in 0.3 ml of the same medium supplemented with glucose at different concentrations and tracer [5-^{3}H]glucose at a specific activity of 0.313 nCi/nmol. After a 60-min incubation, the medium was aspirated and centrifuged at 4°C to remove cell debris. To separate the [^{3}H]water produced from radioactive glucose, columns of Dowex in the borate form were loaded with samples of 0.2 ml of medium and eluted with 3 ml of water (21). The radioactivity in the water eluate was measured by liquid scintillation counting. Glucose utilization was calculated by dividing the measured radioactivity by the specific radioactivity of glucose. Data were expressed per μg of cell DNA, which was measured according to Rymaszewski *et al.* (22).

Northern Blot Analysis of L-Type Pyruvate Kinase mRNA. Cells in 10-cm dishes were lysed in a guanidinium-thiocyanate solution and total RNA extracted (23). Gel electrophoresis of RNA, electrophoretic Northern transfer, and hybridization to a L-type pyruvate kinase cDNA probe (24) were done as described (14).

Insulin Secretion. Cells in 24-well culture dishes were washed twice in Krebs–Ringer solution and incubated at 37°C in 1 ml of the solution supplemented with glucose at different concentrations for 30 min. The medium was withdrawn, centrifuged, and assayed for insulin content by radioimmunoassay. Insulin release was expressed per μg of cell DNA. To measure cellular insulin content, monolayers in replicate wells were overlaid with 1 ml of an ethanol-water-concentrated HCl mixture (750:235:15) and left overnight at 4°C. Cellular material was scraped into the acid-ethanol and briefly centrifuged. The supernatant was assayed for insulin after 1:100 dilution in assay buffer.

RESULTS

Conditional Overexpression of Glucokinase in Insulinoma Cells. The β-cell type insulinoma cell line INS-1 served as parental line for stable transfection of an expression plasmid encoding the reverse tetracycline dependent transactivator of Gossen *et al.* (13). A cellular clone expressing this transactivator, termed INS-r3, was secondarily transfected with a plasmid containing a liver glucokinase cDNA driven by a minimal cytomegalovirus promoter placed under control of the tetracycline operator. Secondary transfectants were screened for high glucokinase expression after culture in the presence of the inducer doxycycline and one clone, termed INS-r3-GK27, was chosen for the present study. The time-course of doxycycline effect on glucokinase activity in INS-r3-GK27 cells is illustrated in Fig. 1*A*. Enzyme activity was induced time-dependently from a basal level of 10 mU/mg of protein to more than 200 mU/mg of protein in 36 h. The dose dependence for doxycycline after 48 h of culture is shown by the immunoblot in Fig. 1*B*. The same samples were assayed for glucokinase activity. A plot of measured glucokinase activities against the optical density of the glucokinase protein bands in the immunoblot is illustrated in Fig. 1*C*. As may be seen, the enzyme activity values were linearily related to the amount of immunodetectable enzyme protein. Collectively, the data indicate that glucokinase activity in INS-r3-GK27 can be set within precise brackets by culturing the cells with well chosen doxycycline doses over defined time intervals. Also important, glucokinase in uninduced INS-r3-GK27 was no higher than in parental INS-1 cells.

Relationship Between Induction of Glucokinase and Glucose Metabolism. The effect of graded increases in glucokinase activity on glucose metabolism was investigated, using the production of [^{3}H]water from [5-^{3}H]glucose to estimate the rate of glycolysis. Cells of the INS-r3-GK27 line were cultured for 14 h in medium with doxycycline concentrations of 75, 150, and 500 ng/ml, or without inducer. The glucose concentration in the culture medium was 2.5 mM during the induction phase. The rate of glycolysis (alternatively referred to as glucose utilization) then was measured over a time period of 1 h, at extracellular glucose concentrations of 2.5, 6, 12, and 24 mM (Fig. 2). In noninduced cells, glucose utilization increased through the range of glucose concentrations, as predicted. More importantly, increasing doses of doxycycline caused a stepwise increase in glucose metabolism at 2.5 and 6 mM glucose. However, at 12 and 24 mM glucose concentrations, glucose utilization tended to plateau at the same level with the two highest doxycycline doses.

The specific activities of glucokinase in cells cultured with the three doses of doxycycline were: 26.8 ± 1.48, 43.2 ± 1.89, and 69.0 ± 3.06 mU/mg of protein, respectively, compared with 8.5 ± 0.67 mU/mg of protein for cells in doxycycline-free medium. This corresponded to mean inductions of 3.2-, 5.2-, and 8.3-fold above basal respectively. The fractional increase of the glycolytic rate was plotted against the fractional increase of glucokinase activity for the two extreme glucose concentrations of 2.5 and 24 mM in Fig. 3*A*. At low glucose, any increment of glucokinase was attended by an increment in glucose utilization of similar magnitude. Therefore, the ratio of the fractional increase in metabolic flux over the fractional

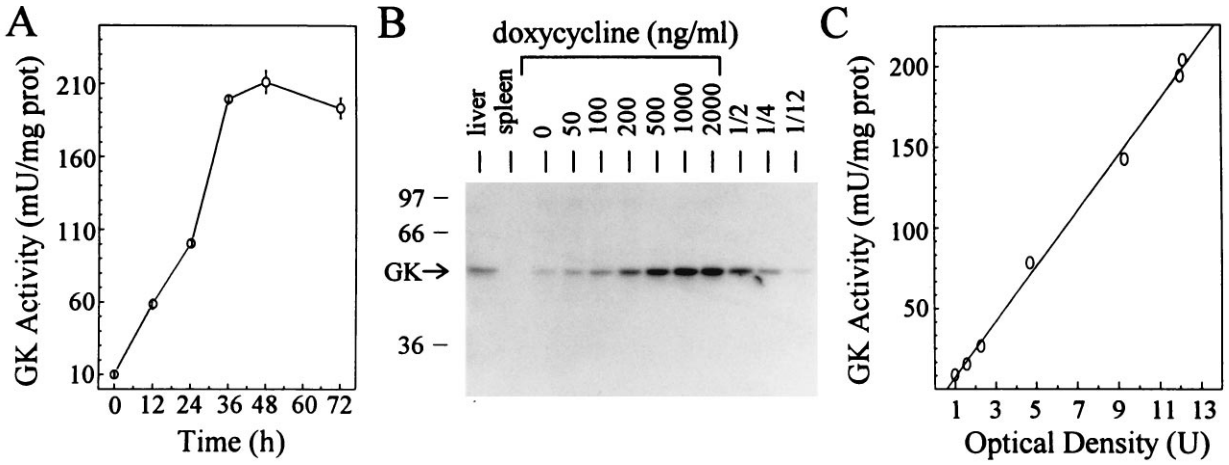

FIG. 1. Time course and log dose-response of doxycycline effect on glucokinase level in INS-r3-GK27 cells. (*A*) Cells were cultured in medium containing 2.5 mM glucose and 1,000 ng/ml doxycycline. They were harvested at the indicated times for enzyme assay of glucokinase activity. (*B*) Cells were cultured with the indicated doses of doxycycline for 48 h. Cell extracts were subjected to SDS/PAGE in a 9% acrylamide gel, transferred to nitrocellulose, and immunoblotted with an antiserum to glucokinase. Lanes were loaded with 10 μg of soluble protein, except the three rightmost lanes, which received (from right) 0.83, 2.5, and 5 μg of protein, respectively, from cells cultured with 1,000 ng of doxycycline per ml. (*C*) Linear regression analysis of glucokinase activity assayed in the same samples, as a function of densitometer units of glucokinase band in the immunoblot.

increase in enzyme, which provides an estimate of the control strength or flux control coefficient of the enzyme (25), was close to 1 for a range of enzyme activities up to 8 times the wild-type level (Fig. 3*B*), indicating that glucokinase is the preponderant rate-limiting enzyme for glycolysis. In contrast, at 24 mM glucose, a 220% increase in glucokinase activity in cells cultured with 75 ng/ml doxycycline was accompanied by no more than a 70% increase in rate of glycolysis (coefficient of 0.31). Moreover, a further enzyme increase of 157% over and above the level noted in cells cultured with 75 ng/ml doxycycline, after culture with 500 ng/ml inducer, caused only a 32% increase in glycolytic flux (coefficient of 0.20). These data indicate that at the supraphysiological glucose concentration of 24 mM, and in the face of even a relatively modest induction of glucokinase, one or several other enzyme steps in the glycolytic pathway assume a major rate-controlling role.

Relationship Between Glucokinase Overexpression and Inductive Response of Pyruvate Kinase Gene to Glucose. The L-type pyruvate kinase gene is transcriptionally induced in response to a rise of the extracellular glucose concentration in the endocrine β-cell (14) and in hepatocytes (26). Although

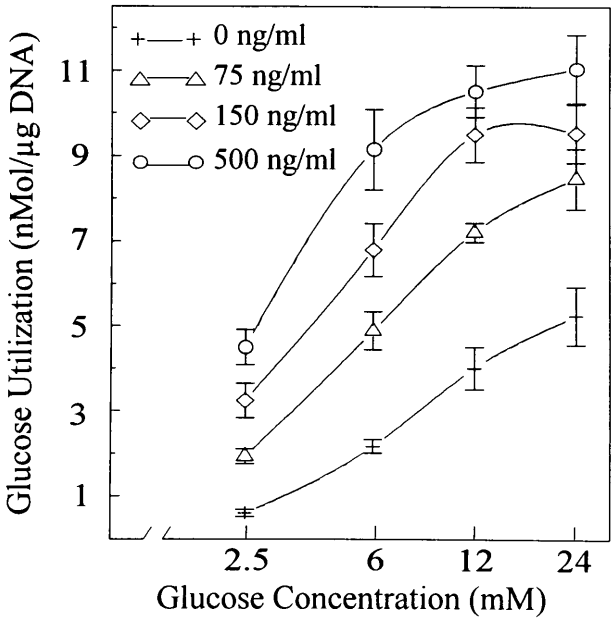

FIG. 2. Effects of various levels of glucokinase induction on rate of glycolysis in INS-r3-GK27 cells. Cells were cultured in medium with 2.5 mM glucose and the indicated doses of doxycycline for 14 h before the experiment. The cells then were incubated with the indicated concentration of glucose and D-[5-^{3}H]glucose for 60 min. Measurements from duplicate wells were performed for each experimental condition. The production of [^{3}H]water was measured, and glucose utilization was calculated from the specific activity of glucose. The data were expressed per μg of cellular DNA. Data are means ± SEM from five independent experiments.

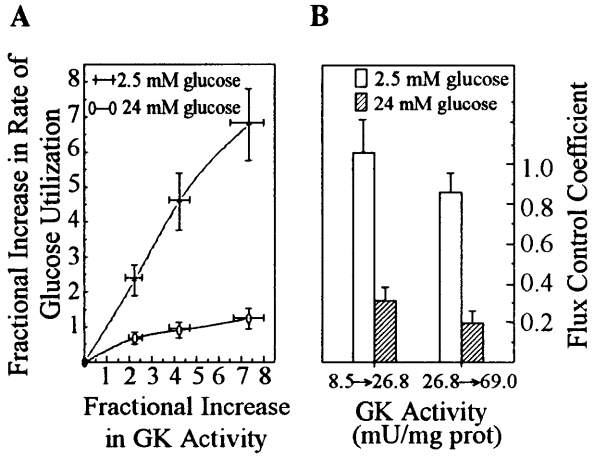

FIG. 3. Flux-control coefficient of glucokinase. (*A*) Glucokinase activity was assayed enzymatically in cell extracts from the experiments in Fig. 2. Fractional increases of glycolytic rate were plotted against fractional increases of glucokinase activity. Fractional increase is the ratio of the difference between induced value minus basal value over basal value. (*B*) Flux-control coefficient of glucokinase at different levels of enzyme induction caused by culture with doxycycline. Flux-control coefficient was calculated as the ratio of fractional increase in glycolytic rate over fractional increase in enzyme activity for two discrete increments in glucokinase activity. The first increment was between cells without inducer and cells cultured with 75 ng/ml doxycycline, and the second between cells cultured with 75 and 500 ng/ml doxycycline. Glucokinase activity was increased from a basal value of 8.5 ± 0.7 to 26.8 ± 1.5 mU/mg of protein after culture with 75 ng/ml doxycycline, and to 69.0 ± 3.1 mU/mg of protein after culture with 500 ng/ml doxycycline. Data obtained at 2.5 mM and 24 mM glucose concentrations are shown. Results are the means ± SEM of five separate experiments.

the signaling mechanism remains incompletely understood, recent studies with hepatoma cells have suggested that the intermediate xylulose 5-phosphate, generated in the pentose phosphate pathway, was a critical metabolite of glucose for the pyruvate kinase gene response (27). The availability of the INS-r3-GK27 cell line allowed us to test the hypothesis that discrete increments in glucokinase activity would allow to amplify the pyruvate kinase gene response to an elevation of extracellular glucose. The cells were cultured with or without doxycycline for 14 h at 2.5 mM glucose, followed by an additional 8 h in medium with 2.5, 6, 12, and 24 mM glucose. In that experiment, glucokinase activity was 26.1, 36.9, and 81.6 mU/mg of protein, respectively with the three doses of doxycycline, corresponding to induction of 2.6, 4.1, and 8.4-fold over basal glucokinase activity, similar to the effects seen in the glucose utilization experiments. Total RNA from the cells was subjected to Northern blot analysis of L-type pyruvate kinase mRNA (Fig. 4*A*). The relative levels of the mRNA at the various glucose and doxycycline concentrations, estimated by phosphorimaging, are plotted in Fig. 4*B*. In absence of doxycycline, the extent of mRNA accumulation at various glucose concentrations was similar to previous results in untransfected INS-1 cells (14). Culture with increasing doses of doxycycline was accompanied by a gradual leftward shift in the dose–response curve for the induction of the pyruvate kinase mRNA by glucose. The maximal responses with the two higher doses of doxycycline were reached at 12 mM glucose, in accordance with the plateau in glucose metabolism shown in the experiments of Fig. 2. These results provide direct evidence for a link between glucose metabolism and pyruvate kinase gene induction, and for the central role of glucokinase in governing both processes. Of interest, a drop in mRNA level was consistently observed with the highest dose of doxycycline between 12 and 24 mM glucose. Both the leftward shift in pyruvate kinase mRNA response and the decline at high glucose, were noted with an independently isolated, glucokinase-inducible cell clone named INS-r3-GK4.

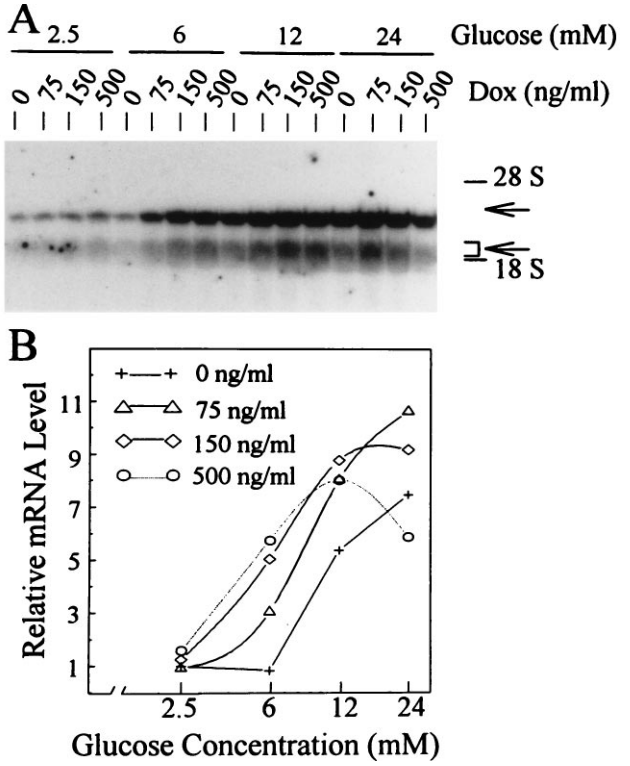

FIG. 4. Induction of L-type pyruvate kinase mRNA by glucose in INS-r3-GK27 cells with various levels of glucokinase. Cells were cultured with various doses of doxycycline at 2.5 mM glucose for 14 h. The resulting levels of glucokinase are given in the text. Culture was continued for 8 h at 2.5 mM glucose or at the indicated glucose concentrations. Total cellular RNA was isolated, and 16 μg of RNA was analyzed by Northern blot with a rat liver pyruvate kinase cDNA probe labeled with ^{32}P. (*A*) Autoradiograph of the blot. Specific mRNA species are 3.1 (upper arrow), 2.2 and 2.0 kb in length (lower arrow), due to multiple polyadenylylation sites. (*B*) Quantification of 3.1-kb mRNA by phosphorimaging. The data are from a typical experiment. The experiment was repeated four times with similar results.

Relationship Between Glucokinase Overexpression and Insulin Secretory Response to Glucose. The cardinal response of the β-cell to a rise in extracellular glucose is insulin secretion. The possibility of modifying this response by controlled overexpression of glucokinase in INS-r3-GK27 cells was investigated. The protocol of induction of glucokinase by three doses of doxycycline was the same as in the previous experiments. Thereafter, insulin secretion was measured over a period of 30 min, in medium containing 2.5 mM of glucose as during the induction phase, or after stepping-up the sugar concentration to 6, 12, or 24 mM (Fig. 5). Cells that were cultured without doxycycline exhibited a 3.4-fold increase in insulin release throughout the range of glucose concentration, 75% of the increment taking place between 6 and 12 mM. This response is similar to that of the original untransfected INS-1 cells (12). Forced expression of glucokinase resulted in a leftward shift of the dose response curve. In cells pretreated with 75 ng of doxycycline per ml, 90% of the maximal insulin response was reached at 6 mM glucose, and the maximal response was not increased over and above that of untreated cells. In doxycycline-treated cells, glucokinase was increased 2.6 times over basal. As seen in Fig. 2, the glycolysis rate at 6 mM glucose in cells with 3-fold induction of glucokinase exceeded or approached the rates found at 12 and 24 mM glucose in control cells. Such concentrations were maximally effective for insulin release. It is therefore not surprising that the plateau for insulin release was reached at 6 mM glucose in cells treated with 75 ng of doxycycline/ml. With the higher doxycycline doses, which caused 4.2- and 6.7-fold inductions of glucokinase respectively, raising glucose beyond 12 mM (150 ng of doxycycline per ml) or 6 mM (500 ng of doxycycline per ml) actually reduced the extent of the glucose effect. The downward inflection of the dose–response curves was reminiscent of the effect noted at the higher doxycycline and glucose concentrations in the pyruvate kinase mRNA experiments.

DISCUSSION

We have implemented the doxycycline-inducible system of Bujard and colleagues (13) for a study of the role of glucokinase as the "glucose sensor" of the endocrine β-cell. A feature of the system particularly advantageous in metabolic investigations is the tight control over the level of the expressed protein. By manipulating the dose of inducer and the time of induction, we could reproducibly target the level of glucokinase within predetermined brackets. Moreover, in the absence of doxycycline, glucokinase activity was the same in INS-r3-GK27 cells as in the parental INS-1 line, indicating no leakage in transcription of the foreign gene in the basal state. It is also important to note that in a control insulinoma cell clone INS-r3-LUC16, with the firefly luciferase gene driven by the tetracycline-dependent transactivator, induction of luciferase by even maximal doses of doxycycline had no effect on glucokinase activity, glucose utilization, pyruvate kinase mRNA induction, and insulin release (data not shown). This demonstrated that doxycycline itself, or the induction of an irrelevant protein, had no effects on the processes under study. The present system thus has allowed the accomplishment of two basic requirements for metabolic control analysis: to set the amount of glucokinase at several discrete levels within a desired range, without untoward unspecific effects; and to

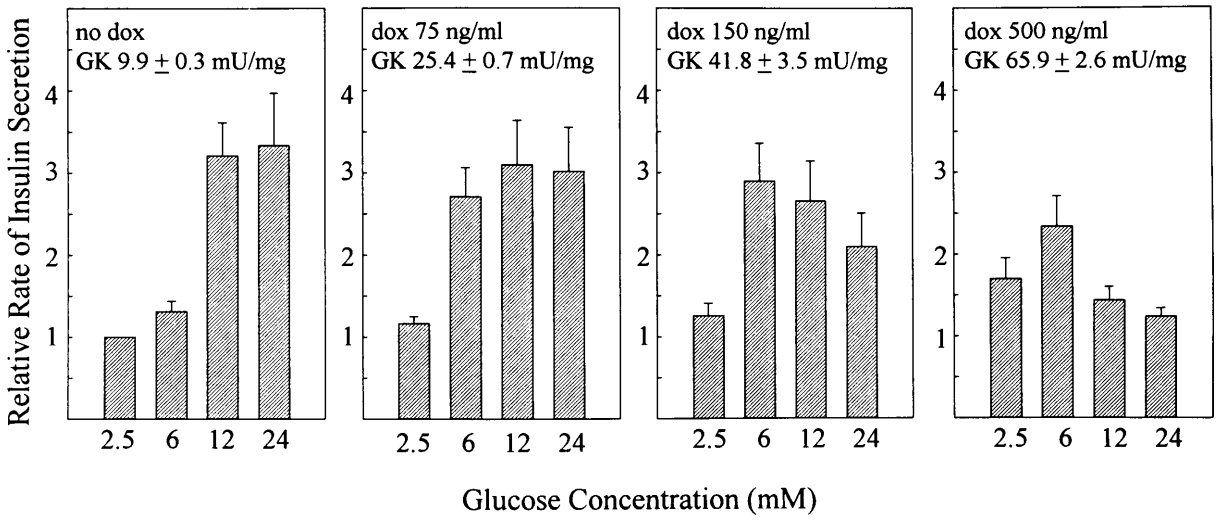

FIG. 5. Stimulation of insulin secretion by glucose in INS-r3-GK27 cells with various levels of glucokinase. Cells were cultured with doxycycline in the presence of 2.5 mM glucose for 14 h. They then were incubated in Krebs–Ringer medium with the specified glucose concentrations for 30 min. Insulin released in the medium was assayed by radioimmunoassay and expressed per μg of cellular DNA content. Data were calculated as ratios of amounts of insulin release over the amount of insulin released by cells cultured without doxycycline at 2.5 mM glucose. This reference value amounted to 0.054 ± 0.010 ng of insulin/μg of DNA. Data represent the means ± SEM of six separate experiments. In each experiment, measurements were performed in duplicates for each experimental condition. Cellular insulin content of cells cultured without doxycycline was 46 ± 6 ng/μg of DNA. Cellular insulin content was decreased 20% in cells cultured with 500 ng/ml doxycycline.

establish a quantitative relationship between these levels and the rates of glycolytic flux at different glucose concentrations.

Two important results concerning the regulatory role of glucokinase in the β-cell type emerge from this study. First, the flux-control coefficient of glucokinase, that is the fractional increase of glycolytic flux divided into the fractional increase of glucokinase activity, was 1 at 2.5 mM glucose and higher than 0.5 at 6 mM glucose, for a range of glucokinase induction comprised between three and eight times the wild-type level. Flux-control coefficients as high as this constitute an exception in metabolic control analysis (28). They provide direct evidence that the glucokinase step is a "bottleneck" for glucose metabolism at physiological extracellular glucose. The term "rate-limiting" can thus be aptly used to designate glucokinase in the β-cell type. The second important observation is that an upper limit in rate of glycolysis was reached at high glucose concentration, such that any increase in glucokinase over and above two times the basal level was essentially unproductive. This indicates that one or several other metabolic steps become rate-limiting under these conditions, as would be predicted from the theory of metabolic control analysis (29).

The induction of specific gene expression and insulin release are physiologically important responses stimulated by a rise of the extracellular glucose concentration. The fact that forced expression of glucokinase resulted in an enhancement of these responses adds further support for a link between them and the rate of glucose metabolism. Signaling for L-type pyruvate kinase gene induction might be mediated by the accumulation of the pentose phosphate pathway intermediate xylulose 5-phosphate (27). Distally, this intermediate appears to activate a specific protein phosphatase (30) and could hypothetically regulate the activity of a factor or cofactor of transcription directly or indirectly. Signaling for insulin release is considered to rely mainly on an increase of the ATP/ADP concentration ratio (31), leading to the closure of potassium channels, membrane depolarization, and calcium entry into the cell. Our data strengthen the notion that glucokinase is the determinant for the generation of the proximal metabolic signals triggering both specific gene induction and insulin release. It should be noted, however, that numerous transduction and effector steps are interposed between the metabolic signals and the end-responses. As has been pointed out, the fact that glucokinase is the "glucose sensor" does not detract from the possibility, indeed the likelihood, that these downstream steps could constitute targets for regulatory mechanisms or pathological disturbances in disease states such as diabetes mellitus (32).

With the conjunction of high glucose concentration and a more than 4-fold induction of glucokinase over the wild-type level, both the L-type pyruvate kinase mRNA and insulin secretion responses were negatively affected. It is intriguing to note that these adverse effects occurred when the fractional increase in glucokinase amount far outstripped the increase in glycolytic rate. We hypothesize that under such circumstances, the rate of conversion of glucose to glucose 6-phosphate in the cell, an ATP-consuming step, exceeds the rate at which glucose 6-phosphate is utilized in downstream metabolic reactions. Hexose phosphates then would accumulate unphysiologically and act as a trap for inorganic phosphate, with deleterious consequences on the energy metabolism of the cell. This proposal remains to be tested. In any event, the present data illustrate the need for precise control over the amount of enzyme in gene transfer experiments applied to metabolic control analysis. Our results suggest that, in this field like in architecture, less can be more.

A final point of discussion concerns the relevance of this work to nontumoral β-cells. The INS-1 cells used as starting material are generally considered to provide a satisfactory, if imperfect, model system (12). The biological features of the INS-r3-Gk27 cells presented here make good physiological sense. It appears very unlikely that they would have arisen *de novo* as a result of tumorigenesis or cell cloning.

We are grateful to Hermann Bujard for generously supplying the plasmids of the doxycycline-inducible system, Nancy Quintrell for the gift of the Tk-hygromycin resistance plasmid, and Axel Kahn for the pyruvate kinase G4 cDNA plasmid. We thank Francoise Assimacopoulos for helpful advice on glucose metabolism experiments and Jean-Claude Henquin for stimulating discussion. This research was supported by Swiss National Science Foundation Grant 32-42207.94.

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