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Additive activation of glucokinase by the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase and the chemical activator LY2121260

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ABSTRACT

The glucose phosphorylating enzyme glucokinase plays a crucial role in stimulus-secretion coupling in pancreatic beta cells and in glucose metabolism in liver. Glucose mediates a shift of the enzyme's conformational equilibrium towards the closed conformation with high glucokinase activity. Further activation of glucokinase is endogenously mediated by interaction with the bisphosphatase domain (FBPase-2) of the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) and can be achieved also by a new class of glucokinase activators (GKA), chemical compounds that might be suited for type 2 diabetes therapy. While FBPase-2 increased only the phosphorylating capacity of glucokinase, the GKA LY2121260 augmented in addition the affinity of glucokinase for glucose. PFK-2/FBPase-2 but not LY2121260 antagonized glucokinase inhibition by the competitive glucokinase inhibitor mannoheptulose at increasing glucose concentrations. Interestingly, an additive activation of glucokinase was observed by use of recombinant FBPase-2 together with LY2121260. This new crucial observation could be confirmed with cellular extracts containing the glucokinase and PFK-2/FBPase-2 proteins. Addition of LY2121260 resulted in a further significant increase in glucokinase activity. Because the glucokinase-PFK-2/FBPase-2 complex was conserved under LY2121260 treatment as shown by size exclusion chromatography a concerted action of both activators towards the closed active glucokinase conformation can be anticipated. Thus, as a result of the additive effect of both activators on glucokinase activity, the largest increase of glucose-induced insulin secretion was observed in the combined presence of PFK-2/FBPase-2 and LY2121260.

1. Introduction

The glucose phosphorylating enzyme glucokinase controls metabolic flux in glycolysis [1,2]. In contrast to the other members of the hexokinase family glucokinase shows a sigmoidal saturation curve and an affinity for glucose in the physiological concentration range between 6 and 8 mmol/l [1–4]. Thus, glucokinase is well suited to play the crucial role of the glucose sensor in the pancreatic beta cell for the control of glucose-induced insulin secretion [1–4]. In liver, with a high expression level, glucokinase is the main regulator of glucose metabolism [5].

Glucose binding induces a global conformational transition of glucokinase [6]. This is the explanation for the positive cooperativity of glucokinase although the enzyme is monomeric [7]. Kinetic models, namely the mnemonical and the slow-transition model have suggested a positive cooperativity of glucokinase [8,9]. A few years ago, two glucokinase conformations were characterized by its crystal structure, a super-opened, catalytically inactive glucokinase conformation and a closed, catalytically active one [10]. In addition there is evidence that further conformational states exist during the transition between the closed and super-opened conformation [10,11]. Three intermediates have been assumed from fluorescence spectroscopy measurements indicating intracellular equilibration of glucokinase conformations depending on the glucose concentration [12].

This glucose-mediated regulation of glucokinase is modulated on the posttranslational level by protein–protein interaction [1,13]. In liver, glucokinase is inhibited by the glucokinase regulatory protein, which binds and shuttles glucokinase at low glucose concentrations to the nucleus [14,15]. The bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) is an activating glucokinase binding partner, both in liver and in pancreatic beta cells [2,13,16–19]. The interaction between glucokinase and the bifunctional enzyme is mediated by a motive in the bisphosphatase domain [16]. Activation of glucokinase through PFK-2/FBPase-2

Abbreviations: GK, glucokinase; PFK-2/FBPase-2, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase.

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increases the maximal enzyme velocity (V_{max}) value of the enzyme, while the affinity for glucose ($S_{0.5}$) remained unchanged [13,17]. In beta cells glucokinase activation by PFK-2/FBPase-2 occurs at high glucose concentrations and potentiates glucose-induced insulin secretion [13,20].

An increase of insulin secretion to an extent that it induces hypoglycemia was observed in patients carrying activating glucokinase mutations [21]. The activating mutations which are located in the hinge domain opposite and spatially remote from the glucose binding site significantly reduce the $S_{0.5}$ of glucokinase for glucose [21–24]. The region, which accommodates the activating mutations [22,24] is the allosteric binding site of small molecules, which were recently found to activate glucokinase [10,25–29]. Their use as blood glucose lowering agents is an attractive therapeutic concept [30]. The action of the different available compounds varies in their activating potency and by the mode how they modify V_{max} and $S_{0.5}$ of glucokinase [10,25–28]. The recently synthesized glucokinase activator LY2121260 raises the V_{max} and decreases the $S_{0.5}$ value [26]. LY2121260 increased insulin secretion in isolated rat pancreatic islets and stimulated glucose uptake in cultured rat hepatocytes [26]. Furthermore, glucose tolerance was improved in healthy rats [26].

To date the interplay between the endogenous activator PFK-2/FBPase-2 and small molecule glucokinase activators such as LY2121260 in the regulation of glucokinase enzyme activity is unknown. The aim of this study was therefore to analyze and compare the molecular mechanisms of glucokinase activation by PFK-2/FBPase-2 and LY2121260.

2. Materials and methods

2.1. Materials

Mannoheptulose and perseitol were from Glycoteam (Hamburg, Germany) and sedoheptulose from Sigma–Aldrich (Steinheim, Germany). The glucokinase activator LY2121260 (2-(S)-cyclohexyl-1-(R)-(4-methanesulfonyl-phenyl)-cyclopropanecarboxylic acid thiazol-2-ylamide) [26] used in this study was kindly provided by Lilly Research Laboratories (Indianapolis, IN). All tissue culture equipment was from Invitrogen (Karlsruhe, Germany) or Greiner-Bio One (Frickenhausen, Germany).

2.2. RINm5F cell culture

Insulin-producing RINm5F cells overexpressing glucokinase (RINm5F-GK cells) were generated by stable transfection of the human beta cell glucokinase cDNA as described previously [17]. RINm5F-GK cells overexpressing PFK-2/FBPase-2 were generated by a second stable transfection of the cDNA for rat islets (RINm5F-GK-PFK-2/FBPase-2 I 4) as described [17]. Cells were grown in RPMI 1640 medium (Biochrom AG, Berlin, Germany) supplemented with 10 mmol/l glucose (Sigma–Aldrich), 10% (v/v) fetal calf serum (FCS) (Biowest, Nuaille, France), penicillin and streptomycin (Biochrom AG) in a humidified atmosphere at 37 °C and 5% CO_2. The medium for RINm5F-GK cells was additionally supplemented with 250 μg/ml geneticin (G418) (Invitrogen) and the medium for RINm5F-GK-PFK-2/FBPase-2 cells with 250 μg/ml G418 and 250 μg/ml Zeocin™ (Invitrogen). For glucokinase activity measurements cells were homogenized in phosphate buffered saline (pH 7.4) and insoluble material was pelleted by centrifugation. The protein concentration was quantified by a Bio-Rad protein assay [17].

2.3. Recombinant glucokinase and FBPase-2 protein

Recombinant beta cell glucokinase was expressed and purified as His_6-tag protein by the pQE30 vector system as described previously [31]. The bisphosphatase domain of rat liver PFK-2/FBPase-2 (amino acid residues 250–470) was expressed and purified as GST-tag protein by the pGEX-6P-1 vector system. The cleavage of the GST-tag was achieved with PreScission protease (GE Healthcare, Freiburg, Germany) as described [19].

2.4. Glucokinase enzyme activity

Glucose phosphorylating activity was measured at different glucose concentrations (1, 1.56, 3.12, 6.25, 12.5, 25 and 100 mmol/l) in recombinant protein solutions or soluble cellular fractions of RINm5F-GK and RINm5F-GK-PFK-2/FBPase-2 (I 4) cells by an enzyme-coupled photometric assay [17]. Pretreatment was performed as indicated. One unit of enzyme activity was defined as 1 μmol glucose-6-phosphate formed from glucose and ATP per minute at 37 °C. Enzyme activities were expressed as units per mg glucokinase protein or cellular protein, respectively. V_{max} were estimated by sigmoidal curve fitting. $S_{0.5}$ values for glucose were calculated from Hill plots.

2.5. Native protein separation and Dot Blotting

Cells were homogenized in phosphate buffered saline (pH 7.4) and insoluble material was pelleted by centrifugation. The supernatant was incubated for 1 h with 25 mmol/l glucose alone or in addition with 10 μM LY2121260. Thereafter for gel filtration chromatography the lysate was loaded on a HiLoad 16/60 Superdex 200 column (GE Healthcare) and eluted with 1 ml/min using ÄKTA® PrimePlus system (GE Healthcare). Resolution of protein separation in the molecular range was calibrated using recombinant glucokinase, bovine serum albumin (70 kDa) (Serva, Heidelberg, Germany), aldolase (158 kDa) and catalase (232 kDa) (Sigma–Aldrich). 15 μl of each fraction was electroblotted to equilibrated polyvinylidene difluoride (PVDF) membranes (Millipore, Schwalbach, Germany) using a 96-well Dot-Blot system (Roth, Karlsruhe, Germany). Nonspecific binding sites of the membranes were blocked by non-fat dry milk overnight at 4 °C. Glucokinase and PFK-2/FBPase-2 immunodetection was performed as described [17].

2.6. Measurement of insulin secretion

MIN6 cells were grown in DMEM medium (Biochrom AG) supplemented with 25 mM glucose, 10% (vol/vol) FCS, penicillin, and streptomycin in a humidified atmosphere at 37 °C and 5% CO_2. Cells were seeded in six-well microplates at a density of 3.5×10^5 cells and grown for three days. Thereafter, cells were transfected with jetPEI (Qbiogene, Montreal, QC, Canada) and 2 μg EYFP, EYFP-PFK-2/FBPase-2 or EYFP-PFK-2/FBPase-2-Mut and grown for further 48 h as described previously [20]. Finally, cells were incubated for 1 h in bicarbonate-buffered Krebs-Ringer solution without glucose supplemented with 0.1% albumin (Sigma–Aldrich) and thereafter stimulated for 1 h with 3 or 10 mmol/l glucose and with or without 10 μM LY2121260. Thereafter 1 ml of the incubation buffer from each well was carefully harvested and gently centrifuged to remove detached cells. In the final supernatants the secreted insulin was measured. Cells were homogenized by sonication in phosphate buffered saline (pH 7.4) and insulin content was measured in soluble fractions. Insulin was measured by ELISA and the protein concentration was quantified by a Bradford protein assay [20].

2.7. Statistical analyses

Data are expressed as means ± SEM. Statistical analyses were performed by ANOVA followed by Bonferroni's test for multiple

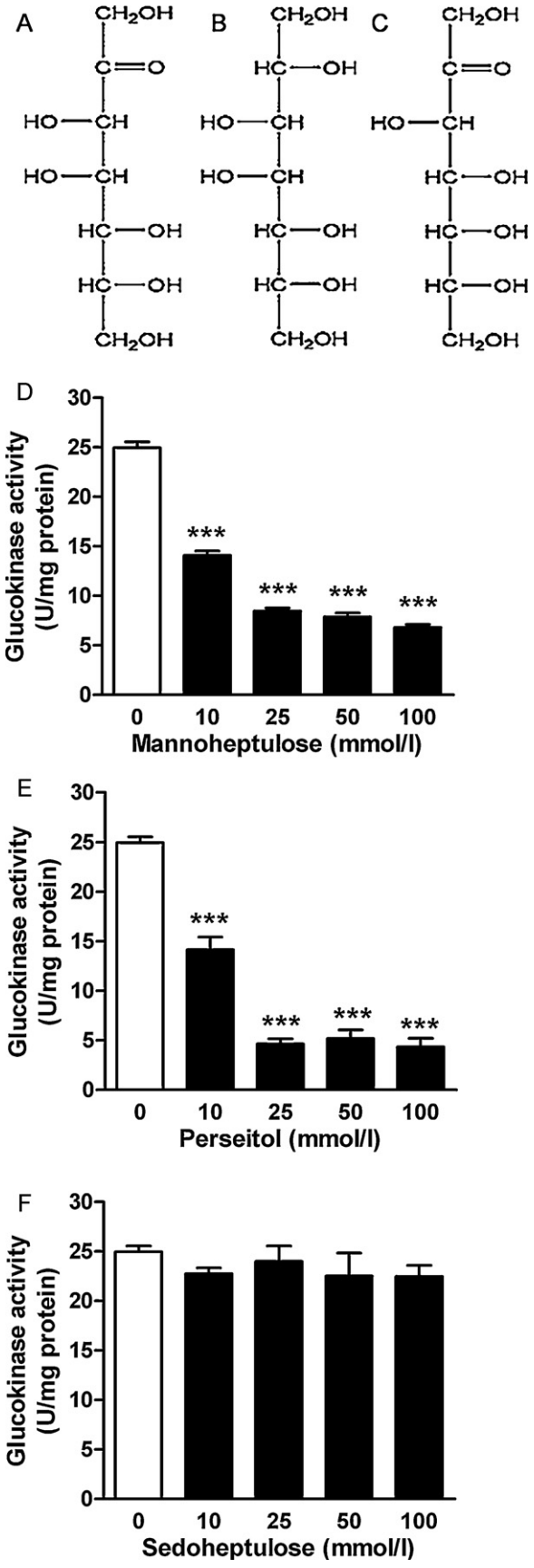

Fig. 1. Inhibition of glucokinase enzyme activities by mannoheptulose, perseitol and sedoheptulose. Chemical structure of mannoheptulose (A), perseitol (B) and sedoheptulose (C). Recombinant beta cell glucokinase was incubated for 5 min with 10, 25, 50 or 100 mmol/l mannoheptulose (D), perseitol (E) or sedoheptulose (F) and after addition of 100 mmol/l glucose enzyme activities were measured spectrophotometrically. Shown are means ± SEM in U/mg protein from 3 independent experiments. ***$p < 0.001$ compared to control (ANOVA/Bonferroni's test).

comparisons using the Prism analysis program (Graphpad Inc., San Diego, CA, USA).

3. Results

3.1. Glucokinase inhibition by mannoheptulose and perseitol

Mannoheptulose and sedoheptulose are epimers. The C7 sugars differ only in the configuration of the hydroxyl group at the 4th carbon atom (Fig. 1A–C). Glucokinase was significantly inhibited by mannoheptulose in a concentration-dependent manner (Fig. 1D). This inhibition was sugar conformation selective since sedoheptulose did not affect glucokinase enzyme activity (Fig. 1F). However, the C7 sugar alcohol perseitol with a configuration of the hydroxyl group at the 4th carbon atom corresponding to that of mannoheptulose showed a significant inhibition of glucokinase (Fig. 1E).

3.2. Activation of glucokinase enzyme activity by LY2121260 in the presence of the inhibitors mannoheptulose and perseitol

Mannoheptulose and perseitol increased the $S_{0.5}$ value of glucokinase for glucose (control, 7.26 ± 0.46 mmol/l) to 17.9 ± 1.57 mmol/l and 15.1 ± 2.53 mmol/l, respectively indicating competitive inhibition (Fig. 2A and B). Treatment of glucokinase with LY2121260 resulted both in an increase in the maximal enzyme velocity (V_{max} 41.8 ± 0.7 U/mg versus 28.5 ± 1.1 U/mg) and an increase in the affinity to its substrate glucose ($S_{0.5}$ 2.51 ± 0.06 mmol/l versus 7.26 ± 0.46 mmol/l) (Fig. 2A–D). LY2121260 was not able to activate glucokinase in the presence of mannoheptulose (V_{max} 18.0 ± 1.1 U/mg versus 16.4 ± 0.9 U/mg). Actually the affinity of glucokinase for glucose decreased further significantly ($S_{0.5}$ 29.9 ± 2.92 mmol/l versus 17.9 ± 1.57 mmol/l) (Fig. 2A). LY2121260 partially increased glucokinase enzyme activity in the presence of perseitol and evoked a significant increase in both the maximal enzyme velocity (V_{max} 25.3 ± 1.0 U/mg versus 14.8 ± 1.3 U/mg) and the affinity to its substrate glucose ($S_{0.5}$ 3.55 ± 0.43 mmol/l versus 15.1 ± 2.53 mmol/l) (Fig. 2B). Sedoheptulose did not counteract glucokinase activation by LY2121260 (V_{max} 41.5 ± 1.3 U/mg versus 23.5 ± 0.5 U/mg and $S_{0.5}$ 2.57 ± 0.16 mmol/l versus 7.46 ± 0.29 mmol/l) (Fig. 2C).

3.3. Simultaneous glucokinase activation by the chemical compound LY2121260 and FBPase-2

Glucokinase interaction with the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) is mediated by the bisphosphatase domain (FBPase-2) [16]. The GK:FBPase-2 complex has a 1:1 stoichiometry [19]. Most likely due to some protein misfolding in the artificial environment a fivefold molar excess of FBPase-2 evoked the highest activation of glucokinase. Incubation of glucokinase with FBPase-2 in a 1:5 molar ratio significantly increased the maximal enzyme velocity (V_{max} 49.8 ± 2.6 U/mg versus 28.7 ± 2.9 U/mg), but did not change the affinity to glucose ($S_{0.5}$ 7.85 ± 0.95 mmol/l versus 7.75 ± 1.85 mmol/l) (Fig. 3A and B). Activation of glucokinase by LY2121260 in the presence of FBPase-2 resulted in a significant further increase of the glucokinase enzyme velocity (V_{max} 62.2 ± 5.6 U/mg versus 40.3 ± 3.1 U/mg) while the $S_{0.5}$ value was comparable ($S_{0.5}$ 1.98 ± 0.26 mmol/l versus 2.21 ± 0.30 mmol/l) (Fig. 3A and B).

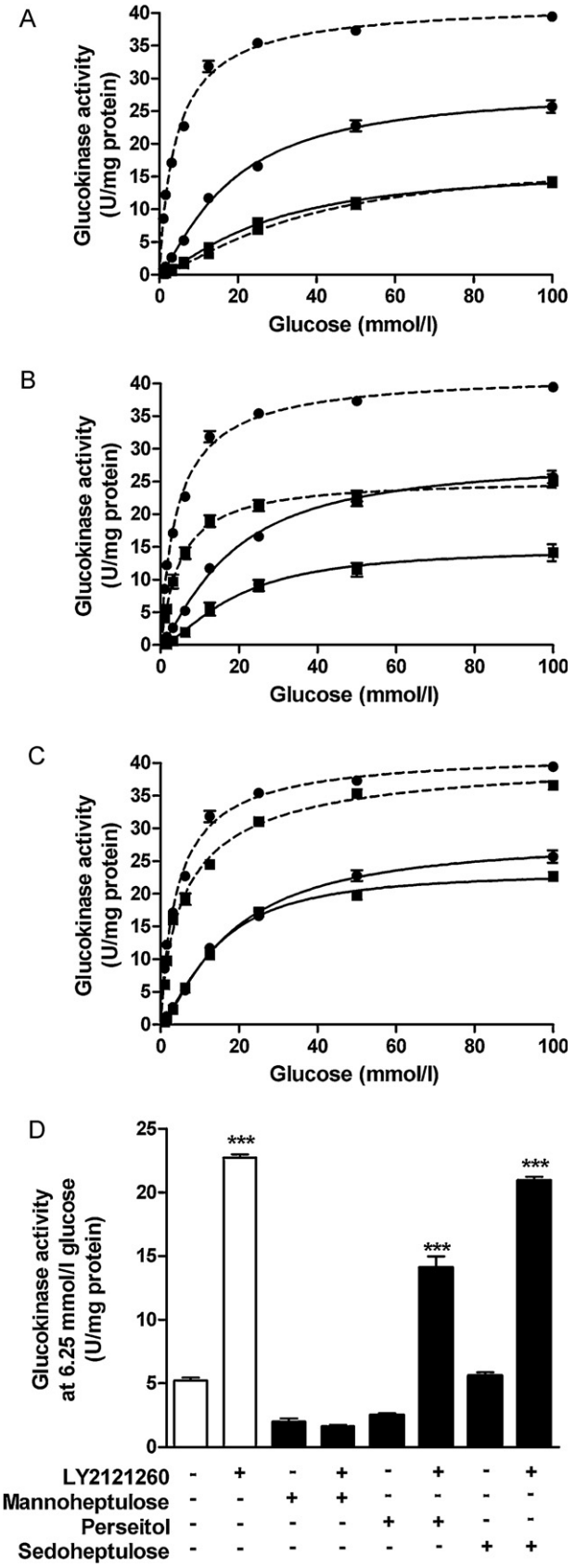

Fig. 2. Effect of the glucokinase activator LY2121260 on glucokinase enzyme activities in the presence of mannoheptulose, perseitol and sedoheptulose. Recombinant beta cell glucokinase was incubated for 5 min without (black circles, solid line), in the presence of 10 μmol/l LY2121260 (black circles, dashed line), in the presence of 10 mmol/l mannoheptulose (A), perseitol (B) or sedoheptulose (C) (black square, solid line) or in the presence of both 10 μmol/l LY2121260 and 10 mmol/l mannoheptulose (A), perseitol (B) or sedoheptulose (C) (black square, dashed line). After addition of the indicated glucose concentration enzyme activities were measured spectrophotometrically. (D) Shown are means ± SEM in U/mg protein from 4 independent experiments. Comparison of glucokinase enzyme activities in the presence of LY2121260 (white bars) and mannoheptulose, perseitol or sedoheptulose (black bars) measured at 6.25 mmol/l glucose. $^{***}p < 0.001$ compared to mannoheptulose, perseitol or sedoheptulose without LY2121260, respectively (ANOVA/Bonferroni's test).

3.4. Glucokinase activation by the chemical compound LY2121260 and PFK-2/FBPase-2 in the presence of mannoheptulose

The stable insulin-producing cell lines RINm5F-GK and RINm5F-GK-PFK-2/FBPase-2 express the glucokinase protein on the same level [13,17]. The presence of PFK-2/FBPase-2 in cell extracts resulted in a significant increase in glucokinase enzyme activity (31% at 6.25 mmol/l glucose) (Fig. 4A and C). Mannoheptulose inhibited glucokinase enzyme activity in both cell extracts. However, RINm5F-GK-PFK-2/FBPase-2 cell extracts showed in the presence of mannoheptulose a higher glucokinase enzyme activity than RINm5F-GK cell extracts (26% at 6.25 mmol/l glucose) (Fig. 4A and C). Thus, at least in part PFK-2/FBPase-2 was able to counteract glucokinase inhibition by mannoheptulose. Treatment with LY2121260 significantly increased glucokinase enzyme activity (59% at 6.25 mmol/l glucose) (Fig. 4B and C). Both activators together evoked a significant further increase in glucokinase enzyme activity compared to PFK-2/FBPase-2 and LY2121260 alone (47% and 27%, respectively at 6.25 mmol/l glucose) (Fig. 4B and C). LY2121260 was not able to activate glucokinase in the presence of mannoheptulose (Fig. 4B and C).

3.5. Glucokinase-PFK-2/FBPase-2 complex formation in the presence of LY2121260

Glucokinase-PFK-2/FBPase-2 complex formation in RINm5F-GK-PFK-2/FBPase-2 cell extracts was detectable as a higher molecular weight fraction by gel filtration chromatography in comparison to RINm5F-GK cell extracts (Fig. 5A and B). Glucokinase was verifiable by Dot-Blot immunodetection analysis in fractions between 50 and 70 kDa and additionally within a molecular range between 130 and 230 kDa. The first peak (50–70 kDa) corresponded to the elution of separated recombinant glucokinase protein in control experiments. In fractions within the peak at higher molecular weight (130–230 kDa) PFK-2/FBPase-2 was identified by Dot-Blot immunodetection analysis with a specific antibody raised against the FBPase-2 domain. Indeed, the glucokinase-PFK-2/FBPase-2 complex was not abolished by treatment with LY2121260 (Fig. 5C).

3.6. Glucose-induced insulin secretion in dependence upon LY2121260 and PFK-2/FBPase-2

Insulin secretion in PFK-2/FBPase-2 expressing MIN6 cells was comparable to control cells at 3 mmol/l glucose. At 10 mmol/l glucose insulin secretion of PFK-2/FBPase-2 expressing MIN6 cells was significantly higher than in control cells (Fig. 6). Treatment with LY2121260 increased insulin secretion at both, 3 and 10 mmol/l glucose in comparison to control cells (Fig. 6). In PFK-2/FBPase-2 expressing MIN6 cells LY2121260 evoked a significant further increase in insulin secretion at 10 mmol/l glucose (Fig. 6). In contrast, expression of a glucokinase binding deficient mutant PFK-2/FBPase-2 protein reduced insulin secretion in MIN6 cells at 10 mmol/l glucose (Fig. 6). Only at 3 mmol/l glucose, but not at 10 mmol/l glucose LY2121260 increased insulin secretion in mutant PFK-2/FBPase-2 expressing MIN6 cells (Fig. 6).

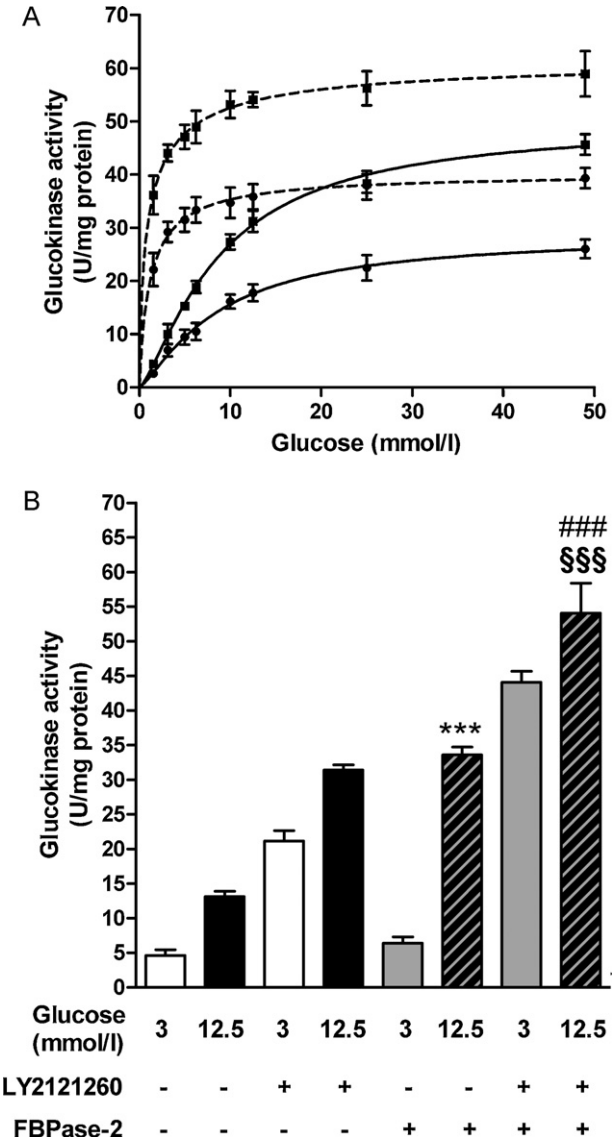

Fig. 3. Effect of the glucokinase activator LY2121260 on glucokinase enzyme activities in the presence of FBPase-2. Recombinant beta cell glucokinase was incubated for 5 min without (black circles, solid line) or in the presence of 10 μmol/l LY2121260 (black circles, dashed line). Recombinant beta cell glucokinase was incubated for 20 min with FBPase-2 in a molar ratio of 1:5 without (black square, solid line) or in the presence of 10 μmol/l LY2121260 (black square, dashed line). After addition of the indicated glucose concentration enzyme activities were measured spectrophotometrically. Shown are means ± SEM in U/mg protein from 3 independent experiments. (B) Comparison of glucokinase enzyme activities measured at 3 (white and gray bars) or 12.5 (black and gray striped bars) mmol/l glucose with or without FBPase-2 and 10 μM LY2121260 as indicated. $^{***}p < 0.001$ compared to control at 12.5 mmol/l glucose; $^{§§§}p < 0.001$ compared to LY2121260 alone at 12.5 mmol/l glucose; $^{###}p < 0.001$; compared to FBPase-2 alone at 12.5 mmol/l glucose (ANOVA/Bonferroni's test).

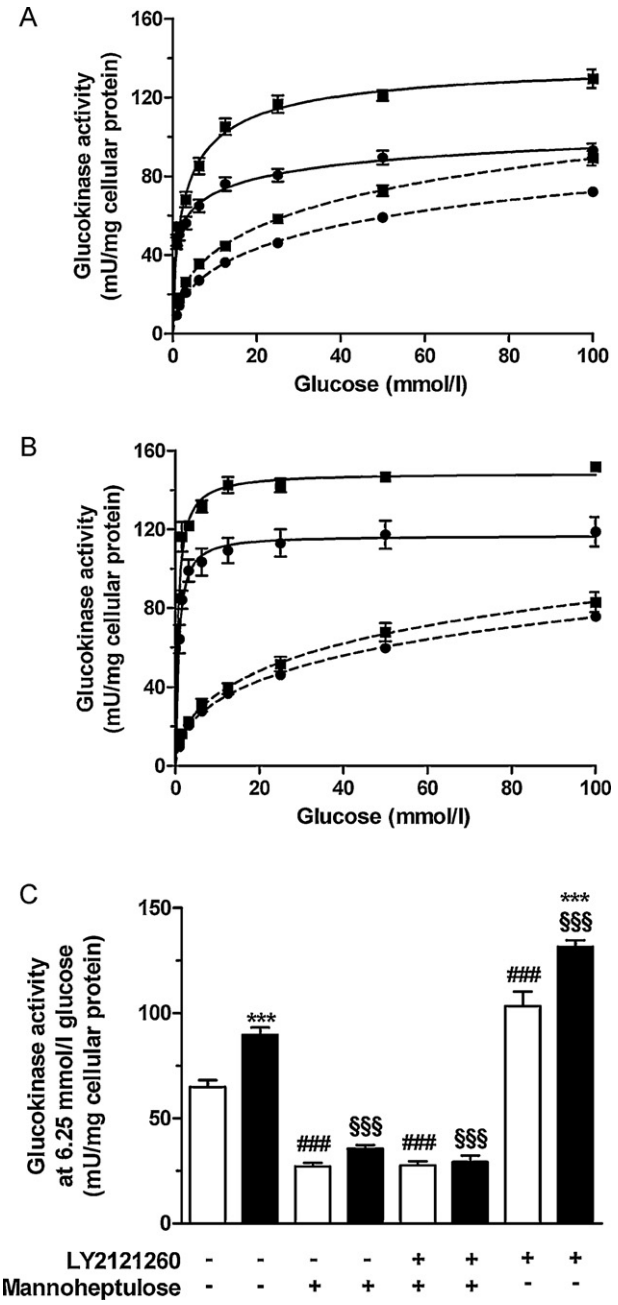

Fig. 4. Effects of the glucokinase activator LY2121260 and mannoheptulose on glucokinase enzyme activities in cell extracts of RINm5F-GK and RINm5F-GK-PFK-2/FBPase-2 cells. Glucokinase enzyme activities were measured spectrophotometrically after sonication of RINm5F-GK cells (circles) and RINm5F-GK-PFK-2/FBPase-2 cells (squares) and 5 min incubation without (A, solid line), in the presence of 10 mmol/l mannoheptulose (A, dashed line), in the presence of 10 μmol/l LY2121260 (B, solid line), or in the presence of both 10 μmol/l LY2121260 and 10 mmol/l mannoheptulose (B, dashed line). Shown are means ± SEM in mU/mg cellular protein from 4 independent experiments. (C) Comparison of glucokinase enzyme activities in cell extracts of RINm5F-GK cells (white bars) and RINm5F-GK-PFK-2/FBPase-2 cells (black bars) in the presence of LY2121260 and mannoheptulose measured at 6.25 mmol/l glucose. $^{###}p < 0.001$ compared to untreated RINm5F-GK cells; $^{§§§}p < 0.001$ compared to untreated RINm5F-GK-PFK-2/FBPase-2 cells; $^{***}p < 0.001$ RINm5F-GK-PFK-2/FBPase-2 cells compared with likewise treated RINm5F-GK cells (ANOVA/Bonferroni's test).

4. Discussion

Glucokinase is the glucose sensor in pancreatic beta cells and the key regulator of glucose metabolism in liver [1–4]. Therapeutically activation of glucokinase is a promising concept to counteract hyperglycemia in individuals with type 2 diabetes. For this purpose several chemical glucokinase activators have been developed recently [10,25–29]. The activators bind to an allosteric site of the glucokinase protein. Thereby they prevent relaxation to the super-opened inactive conformation and increase enzyme activity [10–12,32,33]. However, little is known on how these activators interfere with the endogenous posttranslational glucokinase regulation in beta cells and liver. Glucokinase inhibition through the glucokinase regulatory protein in liver is counteracted by glucokinase activators [32,33]. In this study glucokinase activation by the endogenous interaction partner PFK-2/FBPase-2 and the chemical compound LY2121260 was studied.

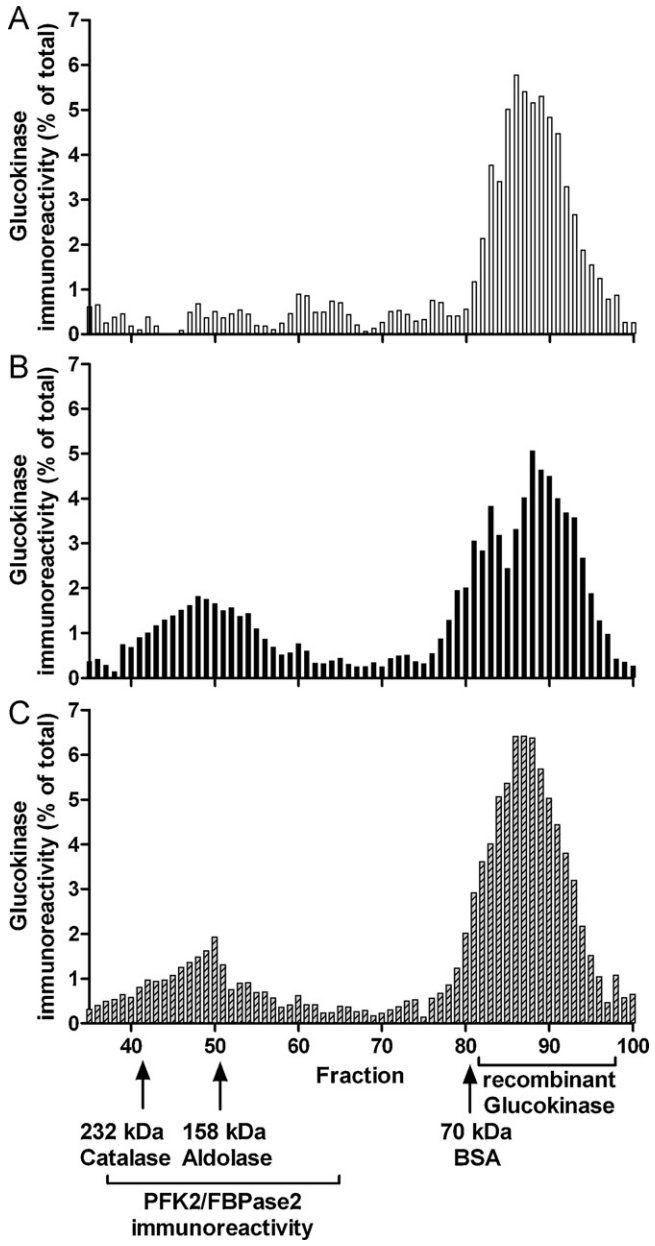

Fig. 5. Glucokinase-PFK-2/FBPase-2 complex formation in RINm5F cells depending on LY2121260. Protein extracts of RINm5F-GK cells (A) and RINm5F-GK-PFK-2/FBPase-2 cells (B and C) were incubated for 1 h with 25 mmol/l glucose alone (A and B) or together with 10 μM LY2121260 (C) and separated by gel filtration chromatography. Glucokinase appearance within the molecular weight fractions was visualized by immunoreactivity and quantified as percentage of total glucokinase protein. Shown are mean values from 3 independent experiments.

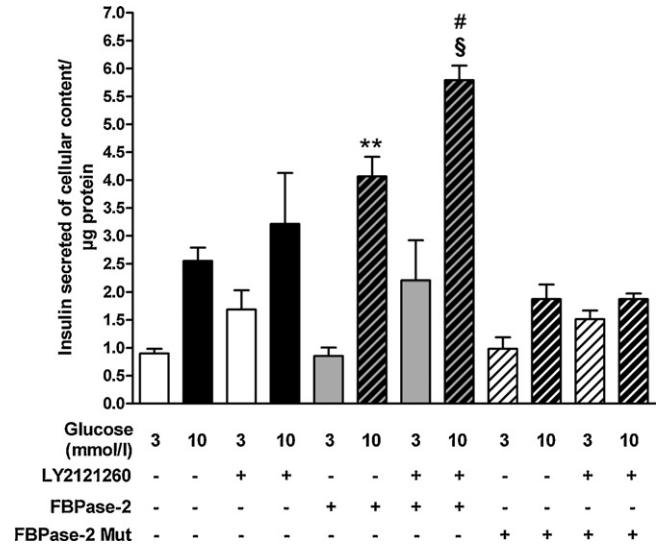

Fig. 6. Effect of the glucokinase activator LY2121260 on glucose-induced insulin secretion in the presence of PFK-2/FBPase-2. Insulin-secreting MIN6 cells were transfected with EYFP (white and black bars), EYFP-PFK-2/FBPase-2 (gray and gray striped bars), or EYFP-PFK-2/FBPase-2-Mut (white striped and black striped bars) and cultured for 48 h. Cells were starved for 1 h and thereafter stimulated for 1 h with 3 (white, gray and white striped bars) or 10 (black, gray striped and black striped bars) mmol/l glucose with or without 10 μM LY2121260 as indicated. Insulin secretion is shown as insulin secreted per cellular insulin content and protein. Data are expressed as means ± SEM of 3 individual experiments. $^{**}p < 0.01$ compared to control at 10 mmol/l glucose; $^{\S}p < 0.05$ compared to LY2121260 alone at 10 mmol/l glucose; $^{\#}p < 0.05$; compared to FBPase-2 alone at 10 mmol/l glucose (ANOVA/Bonferroni's test).

In agreement with previous studies [16,19,20] both the bifunctional enzyme PFK-2/FBPase-2 and LY2121260 increased the maximal velocity of glucokinase. While the low affinity for glucose was maintained during activation through PFK-2/FBPase-2, LY2121260 increased the affinity of glucokinase for glucose. The results of the present study demonstrate for the first time, that the glucokinase:PFK-2/FBPase-2 complex was not diminished by the glucokinase activator LY2121260. Using different approaches we could show that LY2121260 was able to further activate glucokinase in the presence of PFK-2/FBPase-2. This combined effect of the physiological and the pharmacological activator significantly increased insulin secretion at stimulatory glucose concentrations. The importance of PFK-2/FBPase-2 expression for the insulin secretory action of the glucokinase activator LY2121260 could be confirmed with a binding deficient mutant PFK-2/FBPase-2 protein. In the presence of this mutant PFK-2/FBPase-2, insulin secretion was not increased by LY2121260.

The 7-carbon sugar mannoheptulose is a competitive inhibitor of glucokinase [31,34] and like glucose stabilizes the closed glucokinase conformation [35,36]. It has been shown that glucokinase activators not only increase the affinity of glucokinase for its substrate glucose but also for its competitive inhibitor mannoheptulose [27,29]. Thus, in the presence of mannoheptulose glucokinase activators did not increase glucokinase activity and insulin secretion [27,29]. Likewise, LY2121260 did also not activate glucokinase in the presence of mannoheptulose. In contrast, PFK-2/FBPase-2 was able to activate glucokinase with increasing glucose concentrations in the presence of mannoheptulose. This can be explained by the fact, that glucose displaced the competitive inhibitor mannoheptulose from the catalytic site with increasing glucose concentrations. For this process a free conformational equilibration of glucokinase is required, which is supported by PFK-2/FBPase-2, but hindered by LY2121260.

The heptose mannoheptulose has a high affinity to the catalytic center of glucokinase [31]. To prove that the configuration of the hydroxyl group at the 4th carbon atom is crucial for the binding to glucokinase, the effect of sedoheptulose, the epimer of mannoheptulose, was determined. And in fact sedoheptulose did not inhibit glucokinase. Furthermore the action of perseitol, the sugar alcohol of mannoheptulose [37], with the same configuration of the hydroxyl group at the 4th carbon atom on glucokinase was analyzed. Interestingly, perseitol could be identified in the present study as a new inhibitor of glucokinase. LY2121260 was at least in part able to counteract inhibition by perseitol. It can be hypothesized therefore that the alcohol perseitol stabilizes rather an intermediate glucokinase conformation than the closed one. Thus, LY2121260, by promoting the closed conformation, can

induce perseitol release and make the binding site accessible for glucose.

Unfortunately, an analysis of glucokinase inhibition by perseitol in intact cells was not possible, since neither insulin-secreting MIN6 and RINm5F cells nor COS cells took up this sugar alcohol. Thus, it can be assumed that neither the GLUT 1 glucose transporter, mainly expressed in COS cells [38], nor the GLUT 2 glucose transporter, mainly expressed in beta cells [39], can channel perseitol in contrast to mannoheptulose. The fructose transporter GLUT 5 which has been proposed to mediate intestinal uptake of sugar alcohols [38] is not expressed in pancreatic beta cells [40].

In conclusion, this study proves that PFK-2/FBPase-2 facilitates the glucose effect on the glucokinase conformational equilibrium thereby activating the enzyme. In addition, it could be demonstrated that the bifunctional enzyme PFK-2/FBPase-2 and the glucokinase activator LY2121260 have an additive activating effect on glucokinase enzyme activity thereby maximizing the insulin secretory capacity of the beta-cells.

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