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Mutants of glucokinase cause hypoglycaemia- and hyperglycaemia syndromes and their analysis illuminates fundamental quantitative concepts of glucose homeostasis

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Abstract

Aims/hypothesis. Mutations of the glucokinase gene cause hyperglycaemia or hypoglycaemia. A quantitative understanding of these defects of glucose homeostasis linked to the glucokinase gene was lacking. Therefore a database of kinetic variables of wild-type and 20 missense mutants of glucokinase was developed and used in mathematical modelling to predict the thresholds for glucose-stimulated insulin release.
Methods. Recombinant human glucokinase was generated in *E. coli*. The k_{cat}, glucose $S_{0.5}$, ATP K_m, and Hill number of glucokinase were determined. Inhibition by Stearoyl CoA and glucokinase regulatory protein and thermal stability were assayed for all mutants kinetically similar to wild-type glucokinase. A mathematical model predicting the threshold for glucose-stimulated insulin release was constructed. This model is based on the two substrate kinetics of glucokinase and the kinetic variables of the database. It is assumed that both glucokinase gene alleles are equally expressed in beta-cells and that induction of glucokinase occurs as a function of basal blood glucose.
Results. Large changes, varying greatly between mutants were found in nearly all variables. Glucokinase flux at threshold for glucose-stimulated insulin release was about 25 % of total phosphorylating potential in the normal beta-cell and this was used to predict thresholds for the mutant heterozygotes. Clinical data for maturity onset diabetes of the young type linked to the glucokinase gene and familial hyperinsulinaemic hypoglycaemia linked to the glucokinase gene and the glucokinase kinetic data of this study were used to test the model. The model predicts fasting blood glucose between 3 and 7 mmol/l in these cases.
Conclusion/interpretation. A kinetics database of wild-type and 20 mutants of glucokinase was developed. Many kinetic differences were found for the mutants. The mathematical model to calculate the threshold for glucose-stimulated insulin release predicts fasting blood glucose between 3 and 7 mmol/l in subjects with glucokinase gene mutations.
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Keywords MODY-2, glucokinase, glucose threshold, insulin secretion, beta-cell, mathematical model.

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Abbreviations: GSIR, Glucose-stimulated insulin release; DM-gk, maturity onset diabetes of the young type 2 (MODY-2) linked to the glucokinase gene; DM-GK, maturity onset diabetes of the young with abnormal glucokinase protein; HI-gk, familial hyperinsulinemic hypoglycaemia linked to the glucokinase gene; HI-GK, familial hyperinsulinaemic hypoglycaemia with abnormal glucokinase protein; GKRP, glucokinae regulatory protein; β-GPR, beta-cell glucose phosphorylation rate; *h,* Hill Coefficient; $I_{a,}$ activity index; GST-GK, glutathionyl-S-transferase-glucokinase.

The discoveries of linkage to the glucokinase locus on chromosome 7 in autosomal dominantly inherited forms of diabetes mellitus (DM-gk) in 1992 [1, 2] and of hyperinsulinaemia (HI-gk) [3] in 1998 gave a considerable boost to the pancreatic beta-cell glucokinase-glucose sensor concept [4]. The concept had been formulated to explain essential characteristics of glucose-stimulated insulin release (GSIR) and had evolved slowly from its early beginnings in the late '60s [4, 5, 6]. Extensive work with glucokinase knockout mice quickly followed the first reports of glucokinase linkage of diabetes syndromes and fully confirmed the findings in humans [7–9]. Meanwhile

the count of glucokinase mutations causing DM-GK is approaching the 100 mark [10–15], but only one single family with HI-GK has been reported [3].

Precise quantitative understanding of the part that the beta-cell glucokinase-glucose sensor plays in normal glucose homeostasis and the pathogenesis of DM-gk and HI-gk is lacking despite the efforts of several laboratories. First reports confirmed a shift to the right of the GSIR curve and suggested some parallel between the severity of impairment in insulin secretion of the diabetic phenotype and the magnitude of the kinetic changes of some of the mutant glucokinase molecules [16]. This general conclusion was not surprising in view of the basic concept. This early analysis must now, however, be reconsidered because its experimental basis is in part unreliable and advances have been made in fully appreciating the quantitative biochemical and biophysical tenets of the glucokinase-glucose sensor paradigm in glucose homeostasis [17].

There are a variety of reasons to reconsider the relation between kinetic data and their physiological consequences. The most critical are uncertainties about the biochemical and biophysical data base for human wild-type and mutant glucokinase. Most of the published kinetic data on human wild-type and mutant recombinant glucokinase are probably compromised by the inclusion of the functionally significant incidental mutant D158A, which lowers the $S_{0.5}$ for glucose [18–21]. Furthermore, previous studies often did not consider that the recombinant mutant glucokinase protein might be unstable, that the glucose phosphorylation assay is greatly influenced by variables such as pH and ATP-glucose interactions difficult to standardize and that the data processing must account for the cooperativity of glucokinase [17, 22]. The published mathematical modelling attempts of the role of glucokinase in glucose-stimulated insulin release GSIR are limited because they do not consider critical physiological concepts such as the impact on glucose threshold of GSIR, the sigmoidicity of glucokinase-glucose dependency and do not account for the impact on glucokinase activity of changes in the K_m for the second substrate $MgATP^{2-}$ [23]. This combined with limitations of the data base on glucokinase kinetics made it difficult to achieve accurate results from previous modelling attempts.

Our investigation was therefore designed to reconsider the relation between glucokinase function and insulin secretion by taking some new approaches. The rapidity and yield was improved in the production of recombinant human glucokinase using the glutathionyl-S-transferase fusion protein approach. The kinetic analysis of glucokinase was perfected by paying close attention to its unique biochemical and biophysical characteristics. A new minimal mathematical model was formulated describing the relation between beta-cell glucokinase characteristics and GSIR including factors to account for the beta-cell glucose threshold concept, cooperativity of glucokinase with regard to glucose, the interaction of glucokinase with its second substrate $MgATP^{2-}$ and the probability of increased protein lability due to mutations. A sufficiently large sample size was chosen allowing for a critical and comprehensive testing of the model predictions with published clinical data.

Materials and methods

Production of recombinant glutathionyl-S-transferase-glucokinase (GST-GK.) Recombinant human wild-type and mutant beta-cell GST-GK was prepared as described previously [24]. We choose 21 different forms of the enzyme for analysis: the wild-type, the historically significant mutant D158A, nine mutants which had been partially characterized previously [18,21,24,25], nine missense mutations recently identified from DM-gk family studies [15] and the newly described HI-gk mutant V455M [3]. Point mutations were introduced into the pGEX-HIGK expression vector using the QuikChange mutagenesis kit (Stratagene, La Jolla, Calif., USA). The Oligonucoleotides used were synthesized by Research Genetics (Huntsville, Ala., USA). All mutants were transformed into *E. coli* (DH5α strain) and verified by DNA sequencing. Glutathionyl-S-transferase-glucokinase was produced in *E. coli* and then purified from crude extracts as described [24]. Briefly, single-step affinity chromatography was used with glutathione-agarose beads to bind the fusion protein which is then eluted with glutathione. The entire purification procedure is complete in less than 3 h. Purified protein is stored at –80 °C in 30 % glycerol, 50 mmol/l glucose, 5 mmol/l glutathione, 5 mmol/l DTT, 200 mmol/l KCl, 50 mmol/l TRIS buffer (pH 7.4). Protein concentration was determined by the BioRad assay with bovine serum albumin as standard (Hercules, Calif., USA). Purity of the GST-GK preparations was screened for with SDS-PAGE.

Kinetic variables. Glucokinase activity was measured spectrophotometrically using an $NADP^+$ coupled assay with glucose-6-phosphate dehydrogenase as described [24]. The pH of all assays was 7.4, except for assays which assessed the inhibition of glucokinase by glucokinase regulatory protein (GKRP) where a pH of 7.1 was used. The rate of glucose phosphorylation catalysed by glucokinase depends on the concentration of both glucose and ATP. For this reason two protocols for the assays were used. One with glucose as a variable substrate (Protocol A) and one with ATP as the variable substrate (Protocol B). In protocol A 11 glucose dilutions with the second substrate $MgATP^{2-}$ at 5 mmol/l plus 1 mmol/l $MgCl_2$ in excess were used for generating the glucose dependency curve and to calculate V_{max}, glucose $S_{0.5}$ and Hill coefficient (h). In this protocol enzyme was diluted from the stock by buffer devoid of glucose. Because of the cooperative nature of the enzyme, data for glucose dependent activity were fitted to the Hill equation to determine these kinetic variables (equation 1).

Hill equation:

$$V = \frac{V_{max} \cdot [glucose]^h}{S_{0.5}{}^h + [glucose]^h} \quad (1)$$

where v = velocity of the reaction is expressed in terms of the enzyme's turnover number s^{-1} at a given glucose concentration, V_{max} is the maximum velocity, (or k_{cat} in terms of s^{-1}), $S_{0.5}$

is the glucose concentration (mmol/l) at which the reaction is half the maximum, and *h* is the Hill coefficient which describes the cooperativity of the reaction and is unitless. Data fitting was done as described [22]. The concentration of glucose at which the inflection of the dose-dependency occurs (the IP) was calculated as described [26].

The ATP-dependent activity was determined in protocol B using the same assay conditions as in A but with 11 $MgATP^{2-}$ concentrations and glucose at the $S_{0.5}$ of wild-type or the particular mutant. Importantly, the assay was run with 1 mmol/l $MgCl_2$ in excess of $MgATP^{2-}$. The enzyme dilutions from the stock were made in buffer containing glucose at $S_{0.5}$ concentrations. This reaction is characterized by typical kinetics and hence K_m and V_{max} were calculated using the best fit for data with the Michaelis-Menten equation (equation 2):

$$V = \frac{V_{max} \cdot [ATP]}{K_m + [ATP]} \quad (2)$$

The dimensions of the variables are the same as those described for equation 1. [ATP] = concentration of ATP (mmol/l).

We found four mutants to have similar kinetics to the wild-type (A53S, H137R, E300K, V367M) and these were further characterized with GKRP, stearoyl CoA inhibition and thermostability studies. Rat liver recombinant GKRP was kindly provided by Dr. J. Grippo of Hoffmann-La Roche. Stearoyl CoA and GKRP are competitive inhibitors for glucose [18, 27] and the assays were therefore done with glucose at the inflection point of each enzyme so that glucose concentrations are equivalent. Thermal stability of glucokinase was tested in those enzymes with kinetic characteristics similar to the wild-type, and with E70K which had been previously reported to be unstable using protocols as described [22].

Escherichia coli was obtained from Stratagene, cell culture medium from Difco (Detroit, Mich., USA), glutathione-agarose beads were from Sigma (St. Louis, MO, USA) and all other chemicals and reagents, including ATP were obtained from Boehringer Mannheim (Indianapolis, Ind., USA), glucose was purchased from Pfanstiehl (Waukegan, Ill., USA).

Statistical analysis. The results are usually expressed as means ± SEM. The statistical significance of the differences between groups was evaluated by ANOVA using *t* test analysis whenever indicated.

Mathematical modelling methodology. The extensive clinical data base and the present comprehensive information about the glucokinase kinetics of mutant enzymes from DM-gk and HI-gk cases present a unique opportunity for mathematical modelling of GSIR. A minimal model was developed that emanates from earlier concepts about the predominant regulatory role of the beta-cell glucokinase glucose sensor in the process of GSIR and in which the glucose threshold for GSIR is considered the critical variable in glucose homeostasis.

The following assumptions were made: (1) beta-cell glucokinase serves as glucose sensor that controls GSIR; (2) the glucose dependency of beta-cell glucose phosphorylation rate (β-GPR) is adequately defined by the Hill equation. The ATP dependency of β-GPR is defined by an expression based on Michaelis-Menten kinetics. The two equations can be combined to describe two substrate kinetics of glucokinase [28]; (3) the physiological glucose threshold for GSIR is 5 mmol/l which extrapolates to a β-GPR at threshold of 25.4 % of the total capacity of beta-cell glucokinase at physiological $MgATP^{2-}$ concentration of 2.5 mmol/l; (4) the two glucokinase alleles contribute equally to the constitutive basal expression of beta-cell glucokinase protein (GKB) which is assumed to be constant (2GKB); (5) beta-cell glucokinase is inducible by glucose, changing its activity by an adaptive factor of ± 0.2 for ± 1mmol/l change in plasma glucose[29].

The five assumptions can be used to define the relation between β-GPR and the glucose threshold for GSIR.

For two alleles in the beta-cell, total β-GPR is the sum of GPR contributions of allele 1 and allele 2.

For wild-type/wild-type:

$$\beta\text{-GPR} = \frac{[ATP]}{[ATP] + K_m{}^w} \times \frac{k_{cat}{}^w \times G^{hw}}{G^{hw} + S_{0.5}{}^{hw}} \times GKB + \frac{[ATP]}{[ATP] + K_m{}^w} \times \frac{k_{cat}{}^w \times G^{hw}}{G^{hw} + S_{0.5}{}^{hw}} \times GKB \quad (3)$$

Here β-GPR is defined in terms of mol glucose phosphorylated per g of beta-cell mass per s where GKB is the glucokinase content related to each allele of the beta-cells (in terms of mol of enzyme per g of β-cell mass), $k_{cat}{}^w$ the turnover number of wild-type glucokinase (in terms of s^{-1}), G the plasma and beta-cell glucose concentration (mmol/l), *h* the Hill coefficient of glucokinase, $S_{0.5}$ the glucose concentration for half the maxiumum glucokinase activity and w refers to wild-type glucokinase. [ATP] is the intracellular $MgATP^{2-}$ concentration and is assumed to be 2.5 mmol/l for these calculations. $K_m{}^W$ is the ATP concentration required for half maximum glucokinase activity when glucose is in excess. Equation 3 can be rearranged and normalized by dividing both sides by 2 $k_{cat}{}^w$ GKB (= 1.0 or 100 %).

$$\frac{\beta\text{-GPR}}{2k_{cat}{}^w GKB} = \frac{[ATP]}{[ATP] + K_m{}^w} \times \frac{G^{hw}}{G^{hw} + S_{0.5}{}^{hw}} \quad (4)$$

This equation is unitless.

At saturating concentrations of glucose and ATP the β-GPR potential of the beta-cells reaches 100 % in the wild-type homozygote. By defining the GSIR threshold as 5 mmol/l glucose and with 2.5 mmol/l ATP in the cytosol the β-GPR for wild-type/wild-type can be calculated using kinetic variables of wild-type glucokinase from Table 1. This calculation determines that the phosphorylation rate at threshold needs to be 25.4 % of the maximum, that is when glucose and ATP are not rate limiting, to initiate insulin secretion.

Equations 3 and 4 can be reconsidered for the heterozygote situation where the kinetics of the two glucokinase alleles are different (equations 5 and 6). To calculate the GSIR threshold in wild-type/mutant heterozygotes equation 6 needs to be solved for the glucose concentration which results in a phosphorylation rate equal to 25.4 % of the maximum phosphorylating potential of the beta-cell (i.e. equation 4 when glucose is in excess). The kinetic variables for the in vitro experiments in Table 1 (k_{cat} from Protocol B) are used for these calculations. This was done using the Solver function of Excel'98 for each of the mutants listed in Table 1.

$$\beta\text{-GPR} = \frac{[ATP]}{[ATP] + K_m{}^w} \times \frac{k_{cat}{}^w \times G^{hw}}{G^{hw} + S_{0.5}{}^{hw}} \times GKB^w + \frac{[ATP]}{[ATP] + K_m{}^m} \times \frac{k_{cat}{}^m \times G^{hm}}{G^{hm} + S_{0.5}{}^{hm}} \times GKB^m \quad (5)$$

$$\frac{\beta\text{-GPR}}{2k_{cat}{}^w GKB^w} = \frac{0.5\,[ATP]}{[ATP] + K_m{}^w} \times \frac{G^{hw}}{G^{hw} + S_{0.5}{}^{hw}} + \frac{0.5\,[ATP]}{[ATP] + K_m{}^m} \times \frac{G^{hm}}{G^{hm} + S_{0.5}{}^{hm}} \times \frac{k_{cat}{}^m}{k_{cat}{}^w} \times \frac{GKB^m}{GKB^w} \quad (6)$$

Table 1. GST-GK kinetics data base

Mutant	Yield (mg/l)	$S_{0.5}$ (Glucose, mmol/l)	*h*	Inflection Point (mmol/l)	ATP-K_m (mmol/l)	k_{cat}^1 (s^{-1})	k_{cat}^2 (s^{-1})	Mutant
WT[4]	15.9 ± 3.46	8.33 ± 0.27	1.80 ± 0.04	4.12 ± 0.22	0.31 ± 0.03	51.5 ± 4.03	61.7 ± 7.14	WT[4]
A53S	18.5 ± 3.13	8.10 ± 0.22	1.80 ± 0.03	4.07 ± 0.23	0.22 ± 0.04[a]	45.2 ± 5.84	47.8 ± 7.29	A53S
E70K	26.3 ± 1.6[a]	11.5 ± 0.73[c]	1.78 ± 0.07	5.64 ± 0.10	0.36 ± 0.02	43.2 ± 2.26	34.8 ± 4.7	E70K
G80A	18.8 ± 1.27	965 ± 587[b]	0.64 ± 0.04[c]	NO	7.71 ± 1.21[c]	0.06 ± 0.01[c]	0.08 ± 0.01[c]	G80A
H137R	12.9 ± 1.6	7.46 ± 0.38	1.80 ± 0.03	3.74 ± 0.09	0.24 ± 0.04	44.7 ± 5.87	45.3 ± 9.8	H137R
D158A[3]	24.7 ± 3.09	3.67 ± 0.22[c]	1.70 ± 0.03	1.66 ± 0.12	0.33 ± 0.05	52.2 ± 3.69	61.4 ± 2.96	D158A[3]
T168P	18.9 ± 1.77	160 ± 11.1[c]	0.92 ± 0.01[c]	NO	9.33 ± 0.4[c]	0.35 ± 0.04[c]	0.98 ± 0.1[c]	T168P
G175R	26.4 ± 2.63	19.6 ± 0.87[c]	1.74 ± 0.01	9.23 ± 0.35	0.45 ± 0.4[a]	17.1 ± 0.5[c]	23.0 ± 0.53[b]	G175R
V182M	28.0 ± 1.16	34.9 ± 3.4[c]	1.70 ± 0.05	15.7 ± 0.83	0.11 ± 0.01[c]	12.2 ± 0.5[c]	12.3 ± 1.22[b]	V182M
V203A	26.4 ± 3.83	58.4 ± 1.74[c]	1.53 ± 0.01[b]	20.9 ± 1.02	0.88 ± 0.02[c]	12.3 ± 0.8[c]	19.2 ± 0.55[b]	V203A
M210T	7.13 ± 2.18	234 ± 65.2[c]	1.36 ± 0.11[b]	48.4 ± 1.50	5.68 ± 2.61[a]	6.22 ± 2.9[c]	26.4 ± 2.87[a]	M210T
C213R	10.5 ± 2.14	58.4 ± 10.2[c]	1.76 ± 0.04	27.9 ± 5.90	0.85 ± 0.03[c]	14.1 ± 6.6[c]	70.2 ± 13.6	C213R
V226M	18.9 ± 5.56	17.9 ± 2.6[c]	1.41 ± 0.01[c]	5.06 ± 0.68	5.74 ± 1.32[c]	42.7 ± 7.37	61.8 ± 5.27	V226M
T228M	21.7 ± 5.28	NO	NO	NO	NO	< 0.02	< 0.02	T228M
G261R	2.09 ± 0.6[a]	118; 130[6, c]	2.20; 1.90[6, a]	74.4; 72.5[6]	3.83 ± 0.78[a]	1.04; 1.51[6, c]	19.4 ± 0.9[b]	G261R
E300K	6.94 ± 0.32[a]	10.3 ± 0.9[b]	1.85 ± 0.02	5.37 ± 0.58	0.46 ± 0.01[a]	33.8 ± 4.17[a]	59 ± 9.38	E300K
L309P	0.27 ± 0.10[a]	9.46 ± 0.62	1.92 ± 0.01	5.10 ± 0.33	0.58 ± 0.09[a]	1.10 ± 0.34[c]	2.92 ± 1.31[b]	L309P
S336L	24.7 ± 5.19	5.42 ± 0.2[c]	1.13 ± 0.14[c]	0.52 ± 0.58	21.7 ± 3.29[c]	1.03 ± 0.15[c]	5.42 ± 0.45[b]	S336L
V367M	23.1 ± 6.40	7.56 ± 0.58	1.80 ± 0.06	3.77 ± 0.50	0.31 ± 0.04	58.1 ± 7.21	65.2 ± 6.44	V367M
K414E	17.4 ± 4.40	12.3 ± 3.30[a]	1.67 ± 0.11	5.57 ± 2.30	1.73 ± 0.2[c]	11.0 ± 3.33[c]	46.7 ± 5.25	K414E
V455M[5]	8.3 ± 1.88[a]	3.18 ± 0.17[c]	1.66 ± 0.03[b]	1.38 ± 0.11	0.25 ± 0.02[a]	48.6 ± 3.72	59.6 ± 7.07	V455M[5]

Most data represent means ± SEM from three separate enzyme expressions. [a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$ compared to wild-type. The inflection point is a mathematical derivative of glucose $S_{0.5}$ and was therefore not submitted to statistical analysis

[1] Protocol A; [2] Protocol B; [3] Incidental Mutant; [4] $n = 9$; [5] $n = 11$; [6] $n = 2$; NO, not obtainable

It is assumed in this formulation that the amount of glucokinase enzyme per g beta-cell mass is constant (i.e. $GKB^m/GKB^w = 1$) and that exact knowledge about the beta-cell glucokinase content and total pancreatic beta-cell volume is not required to define the relation between β-GPR and threshold for GSIR (note, however, the discussion of instability mutants). It is also assumed that the beta-cell mass could be highly variable between subjects but will have little effect on the threshold which is regulated by intracellular events. Overall baseline release will probably be greater in subjects with relatively large beta-cell mass which increased numbers or volumes of beta-cells and this could influence the magnitude of the secretory response, but probably not the level at which insulin secretion starts to increase from baseline, that is the threshold.

Using this technique, predicted glucose thresholds were calculated for each wild-type/mutant heterozygote recorded in Table 1. To be able to rank the mutants in terms of relative phosphorylating potential we have defined an activity index (I_a), derived from the kinetic variables, k_{cat}, $S_{0.5}$, *h*, [ATP] and the K_m for ATP (Expression 7). Intracellular ATP needs to be included in this index because at its physiological concentration of about 2.5 mmol/l it is rate limiting for wild-type and most of the mutants and hence impacts on the activity of the enzyme in the beta-cell.

$$\text{Activity index} = \frac{[ATP]}{[ATP]+K_m} \times \frac{k_{cat}}{S_{0.5}{}^{h}} \ (s^{-1} \times l/mmol^h) \qquad (7)$$

The Ia values for each of the GST-GK enzymes are presented in Table 2. To normalize these values wild-type I_a is defined as 1 and the values for the mutants are presented relative to wild-type in column 2. This normalized activity index is unitless. Note that this minimal model does not address basal insulin release (whatever its biochemical basis) contributing as much as 50% of total daily insulin production, does not assess the swiftness and magnitude of GSIR when blood glucose exceeds the threshold level and does not address peripheral insulin responsiveness. Normality of these variables is an absolute requirement for the present limited model of glucose homeo-

Table 2. Actual and normalized index of activity of wild-type and mutant glucokinase

Glucokinase	Index of activity, $I_{a\text{-}Mt}$[a]	$I_{a\text{-}Mt}$ normalized to wild-type[b]
wild-type	1.21	1.00
A53S	1.02	0.84
E70K	0.37	0.31
G80A	0.00024	0.00020
H137R	1.11	0.92
D158A	5.95	4.92
T168P	0.0022	0.0018
G175R	0.12	0.10
V182M	0.03	0.02
V203A	0.03	0.03
M210T	0.0048	0.0040
C213R	0.03	0.03
V226M	0.33	0.27
T228M	< 0.0001	< 0.0001
G261R	0.00050	0.00041
E300K	0.67	0.55
L309P	0.03	0.03
S336L	0.09	0.07
V367M	1.52	1.26
K414E	0.39	0.32
V455M	7.58	6.27

Expressed in terms of $s^{-1} \times l/mmol/l^{ha}$, unitless[b]
Mt, human mutant glucokinase

stasis, a requirement evidently fulfilled in cases with DM-gk and HI-gk syndromes [3,15].

Results

Yields of recombinant glucokinase, kinetic variables and thermolability. At least three preparations of wild-type and each mutant GST-GK were purified with yields ranging from 0.27 to 28.0 mg/l (Table 1). Despite a wide range in yields, the yields for a particular mutant were highly reproducible. All 21 GST-GK proteins were found to be essentially pure as indicated by the presence of a single band at 75 kDa on phast gel (Amersham Pharmacia Biotech, Piscataway, N.J., USA) electrophoresis (not shown).

The kinetic variables (k_{cat}, glucose $S_{0.5}$, *h*, and ATP K_m) for wild-type and mutant GST-GK are summarized in Table 1. Two different k_{cat} values are recorded, one calculated from data obtained with protocol A and the other based on those from protocol B. Four of the mutants, A53S, H137R, E300K and V367M, show kinetic variables that are essentially the same as wild type. For the other 16 mutants a wide variety of kinetic differences from wild type exist. Glucose $S_{0.5}$ values range from as low as 3.2 mmol/l for V455M, and 3.7 mmol/l for D158A to as high as 235 mmol/l for M210T or even close to 1 mol/l for G80A. Activity is very low in G80A, T168P and T228M despite good protein yields. This low activity makes analysis of data difficult and the kinetic results are therefore only approximations. The Hill numbers for G80A, T168P, M210T, V226 M and S336L are considerably less than wild type, two of them close to 1.0. It is remarkable that the lowest Hill numbers coincide with the highest ATP K_m values (G80A, T168P, M210T, V266 M and S336L). Differences can be seen between the k_{cat} values of protocol A and the k_{cat} values of protocol B for many of the enzymes (i.e. M210T, C213R, G261R, E300K and K414E). In protocol A, used to determine the glucose $S_{0.5}$, the $MgATP^{2-}$ was 5 mmol/l and this could have been rate limiting in the cases with mutants with high ATP K_m explaining some of the differences. In protocol B, used to determine the ATP K_m, this problem was eliminated by selecting equivalent glucose concentrations and setting the glucose at the $S_{0.5}$ of each mutant. Large differences exist, however, between the two k_{cat} values even for enzymes with relatively low ATP K_m, such as C213R and E300K. The critical difference between the two methods was that the pre-incubation dilutions of the enzyme in B were made with $S_{0.5}$ concentrations of glucose but without glucose in protocol A. Glucose could be stabilizing the enzyme and therefore result in greater activity in protocol B.

Using factor XA to cleave the GST from the glucokinase, wild-type, S336L and V455M were prepared in a pure form. Kinetic profiles for these three enzyme were the same as those for the GST-GK preparation, reconfirming previous observations that the GST tag does not affect glucokinase kinetics (data not shown).

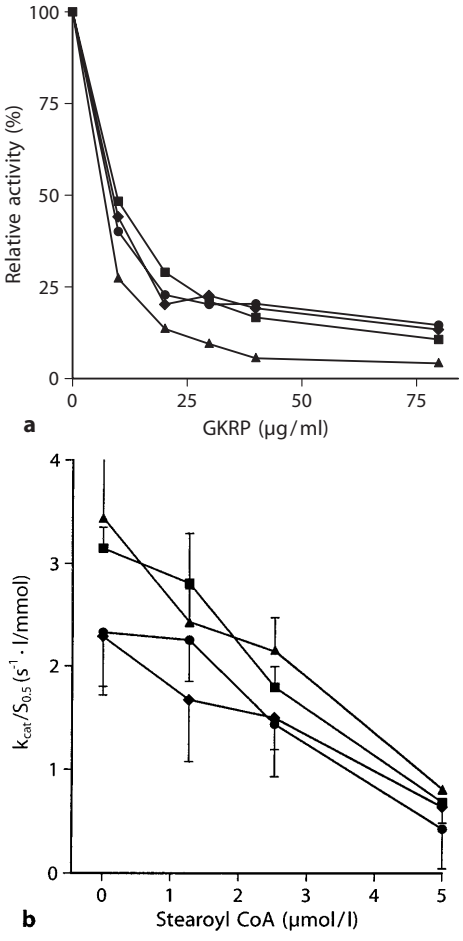

Fig. 1a, b. Inhibitory effects of GKRP and Stearoyl CoA on wild-type GST-GK, A53S, H137R and V367M. **a** Inhibition of GKRP was measured spectrophotometrically with an NADH coupled assay (KCl 25 mmol/l, HEPES 100 mmol/l, $MgCl_2$ 0.5 mmol/l, BSA 0.1 %, ATP 1 mmol/l, DTT 1 mmol/l, NADH 0.4 mmol/l, pyruvate kinase 15 μg/ml, LDH 10 μg/ml, P-enolpyruvate 10 mmol/l and fructose-6-phosphate 1 mmol/l). The assay is run with 1 μg/ml of GST-GK at pH 7.1. One experiment was done in each case. Note the E70K, E300K gave similar results (not shown). **b** Effects of Stearoyl CoA: relative activity values are the mean of three experiments. Assays were $NADP^+$ coupled as described in the Methods. Stearoyl CoA is added to final concentrations of 2.5, 5 and 10 μmol/l. Note that E70K, E300K gave similar results (not shown). (—■—WT; —◆— A53S; —●— H137R; —▲— V367M)

To search for possible explanations of the phenotypes not explained by kinetic changes the four mutants with kinetics profile similar to wild-type (A53S, H137R, E300K, V367M) had inhibition with GKRP and Stearoyl CoA determined (Fig. 1; E300K not shown). No difference in inhibition of wild-type and the mutants was found with either inhibitor. Thermo-

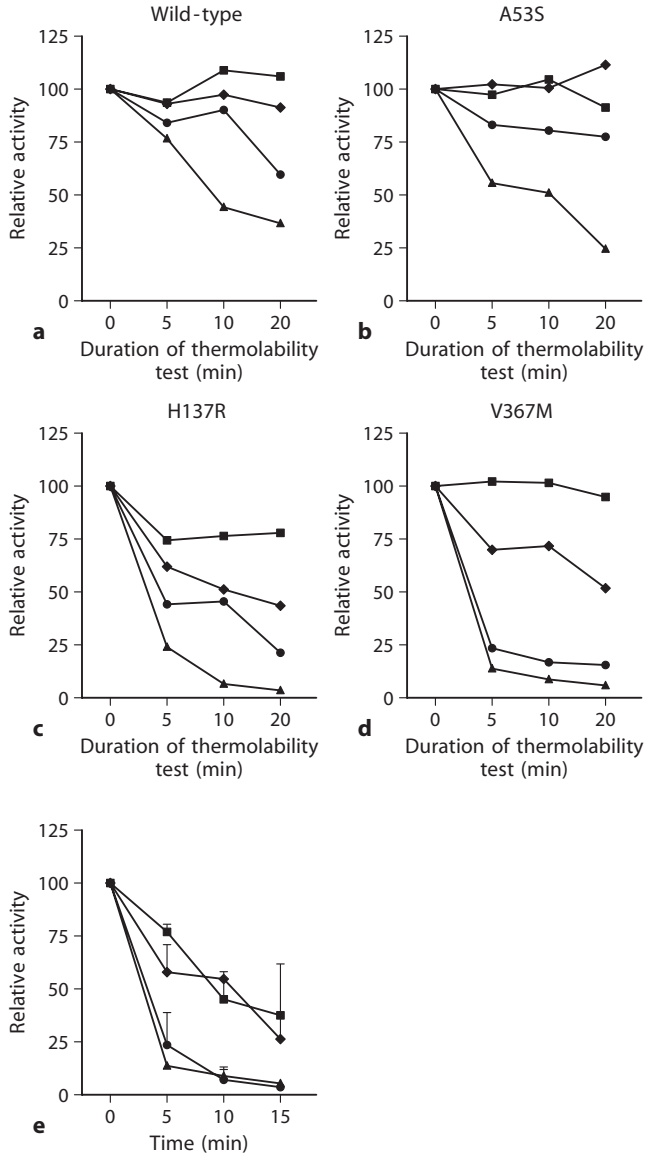

Fig. 2 a–e. Effect of temperature on the stability of wild-type GST-GK, A53S, H137R and V367M. Enzyme stock solutions were diluted in storage buffer to 300 μg/ml. Enzyme was further diluted to 100 μg/ml in 1/3 storage buffer (resulting in 16 mmol/l glucose and 10 % glycerol) for incubation in a water bath at 37, 41, 45 and 49 °C for 0, 5, 10 and 20 min. Glucokinase activity was determined spectrophotometrically as described in Methods. Relative activity values are means of three experiments. —■— 37 °C; —◆— 41 °C; —●— 45 °C; —▲— 49 °C. For clarity SEM are not included on all figures. They are shown in **e** for all 4 proteins using results at 49 °C as an example. At time zero the average k_{cat} for wild-type, A53S, H137R and V367M was 49.1, 45.2, 44.7 and 58 s^{-1} (—■— GK control; —◆— A53S; —●— H137R; —▲— V367M)

lability of these mutants was also assessed. Thermal stability of three of these mutants can be seen in Fig. 2. Both H137R and V367M have decreased activity with increasing time of incubation and with increasing temperature compared with the wild-type, consistent with thermal instability. The behaviour of A53S is, however, essentially the same as that of the wild-type. It was confirmed in this series of experiments that E300K is much more thermolabile than the wild-type but the behaviour of E70K in the temperature titration assay was found to be no different from wild-type in contrast to our previous findings (not shown)[22].

Predicting post-absorptive blood glucose concentrations and glucose thresholds for insulin release from kinetic data. Glucokinase is the known glucose sensor in the beta-cell and determines at what glucose concentration, or threshold, insulin secretion begins to respond to a rise of plasma glucose. To predict the impact of glucokinase mutations on the beta-cell glucose threshold we have used a minimal mathematical model. It uses the Hill equation to incorporate glucose kinetics, as well as an expression to account for the first order ATP kinetics. The model incorporates all the known kinetic variables of the wild-type and mutant glucokinase enzymes and is based on the calculation that about 25 % of the total glucokinase phosphorylating capacity of the beta-cell is needed to initiate insulin secretion, if wild-type/wild-type threshold is defined as 5 mmol/l glucose.

Using this method, predicted glucose thresholds are calculated to range from 2.9 mmol/l for the hypoglycaemia mutant (V455M) to nearly 10 mmol/l for enzymes with minimal activity such as G80A, T168P, M210T, T228M and G261R and are shown in Fig. 3a. Wild-type threshold is at 5 mmol/l as defined. Each mutant is plotted as a function of its normalized activity index (Ia) as defined in the Methods (Table 2). The four mutants kinetically similar to wild-type, have glucose thresholds clustered close to wild-type. Of these, H137R, E300K and V367M were found to be thermally unstable in vitro. Instability presumably leads to greatly decreased activity in the beta-cells and higher glucose threshold. If instability were incorporated into the model and if it were assumed to cause total loss of the mutant glucokinase's contribution to glucose phosphorylation (i. e. $GKB^m/GKB^w << 1$), the thresholds for these three mutants would lie on the threshold curve to the right of the figure. Mutants with partial loss of activity due to instability would lie somewhere in-between. The E300K mutant has now been shown to be very labile not only in vitro, but when expressed in β-HC cells and this observation shows that it is not unreasonable to extrapolate from the results in the cuvette to the cytosol of the beta-cell [30]. At this point no statistically significant kinetic or other differences from wild-type were identified to explain the DM-gk phenotype of the A53S mutant. The normalized glucokinase activity index for A53S is 0.84 compared with 1.0 for the wild-type, which results in a threshold of 5.35 mmol/l glucose, slightly greater than the

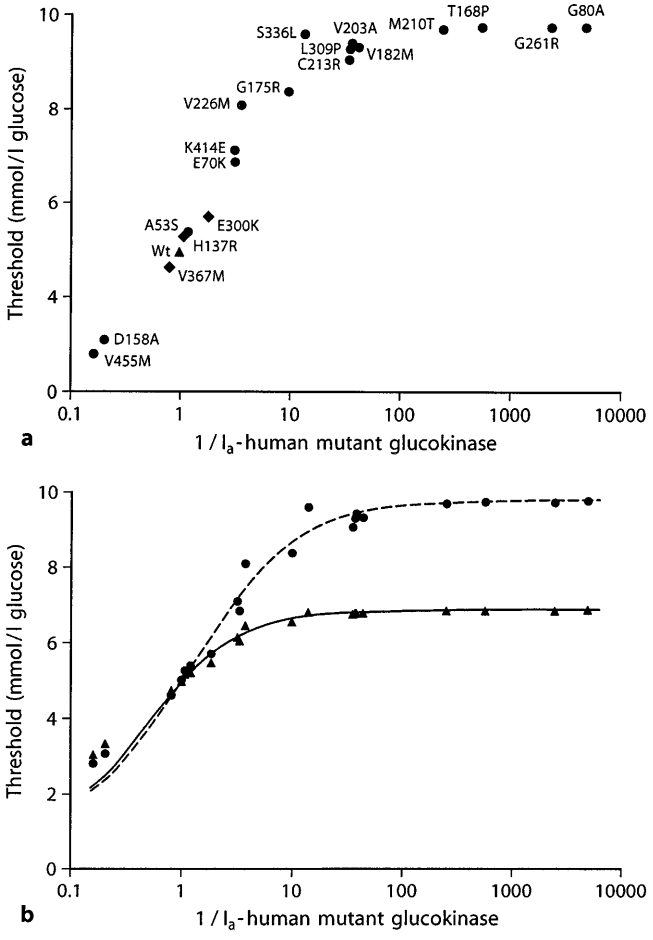

Fig. 3. **a** Beta-cell thresholds for GSIR in wild-type homozygotes and heterozygotes with 20 actual and incidental GST-GK mutants. The glucokinase activity index is an expression of glucokinase capacity and incorporates all kinetic variables (see Table 2 and Equation 7). A53S, H137R, E300K and V367M have glucose threshold clustered close to wild-type (Wt) threshold GSIR of 5 mmol/l. Of these, the last three are thermal instability mutants. If instability occurs in vivo and was included in the model, results would lie to the right of the figure. T228M has an activity index smaller than 0.0001 and lies to the right of this figure.(● glucokinase with low or high activity; ◆ glucokinase with instability; ▲ wild-type glucokinase. **b** Thresholds of GSIR for natural glucokinase mutants, wild-type and theoretical mutants in the adapted and non-adapted state. The theoretical line is a continuum for assumed mutants with $S_{0.5}$, ATP K_m and *h* same as wild-type but a change in k_{cat} over a wide range. The method for adaptation for theoretical and GST-GK mutants is as described in the Methods Section. (Actual glucokinase mutants no adaptation ●; actual GK mutants adaptation ▲; Theoretical k_{cat} mutants ---, —

5 mmol/l of wild-type, but probably not high enough to have been detected as clinically abnormal.

Adaptation, or increased glucokinase activity with persistently increased glucose, has been shown in cultured rat islets [29] and glucokinase activity increased by a factor close to 0.2/mmol/l glucose. Adaptation was also speculated to occur in situ on the basis of clinical studies [23]. Knowing the predicted thresholds from the model in the non-adapted state, assumptions can be made about adaptation of the wild-type allele in vivo. There is, however, not enough information on the mutant glucokinase behaviour to say whether it too would adapt. The mutant allele was therefore ignored in this data processing and the wild-type allele was relied on to fully adapt. For example, for a heterozygote with a mutant allele of minimal activity the predicted glucose threshold without adaptation taking place would be 9.8 mmol/l. There is good evidence that exposed long-term to increased glucose the wild-type allele will increase its basal concentration of 1.0 by a factor of 0.2 for every mmol/l glucose. Increased islet glucokinase activity in turn reduces the threshold for GSIR and eventually an equilibrium is reached. The experimentally derived factor of 0.2 glucokinase/mmol glucose change can be built into the model in equation [6] and successive approximations can be used to predict the final adapted threshold. Predicted thresholds with adaptation for the mutants studied here are presented in Figure 3b. The thresholds for the mutants can be seen to sit very closely along the line for a theoretical mutant with a change in k_{cat} only. After adaptation it is seen that the threshold varies little between mutants. For mutants with minimal activity this predicts an adapted threshold of about 7.0 mmol/l. This value is similar to clinical data in the literature [15, 17, 31, 32] (Table 3). Note that some patients with DM-gk mutants not investigated here carry only one functional allele because they have substantial deletions and early termination events resulting in a mutant activity index of less than 0.0001. Including these would have greatly increased the database. The mean value of 6.7 mmol/l glucose for GSIR threshold accounting for adaptation is remarkably close to the average fasting glucose reported for DM-gk in those studies listed in Table 3. These data also support the choice of 5 mmol/l GSIR threshold in the wild-type homozygote.

Obviously, the limitation of any model is the validity of the assumptions made. For this reason we have looked at the impact of the two main assumptions (Fig. 4). The first is that about 25% of total glucokinase phosphorylating capacity is needed to initiate insulin secretion (Fig. 4a). The hypoglycaemia mutant V455M and a MODY mutant C213R were taken as examples and thresholds calculated using a range from 15–35% of total glucokinase activity in the adapted and the non-adapted state. It can be seen that over the range of 15–35% there is about a 2 mmol/l difference in the threshold of the wild-type. The effects of increasing glucokinase activity at threshold is notable when adaptation is ignored. For example, the threshold for C213R increases to 15 mmol/l with 35% glucokinase activity at threshold and no adaptation. Figure 4b shows the effect of manipulating the second assumption that adaptation of

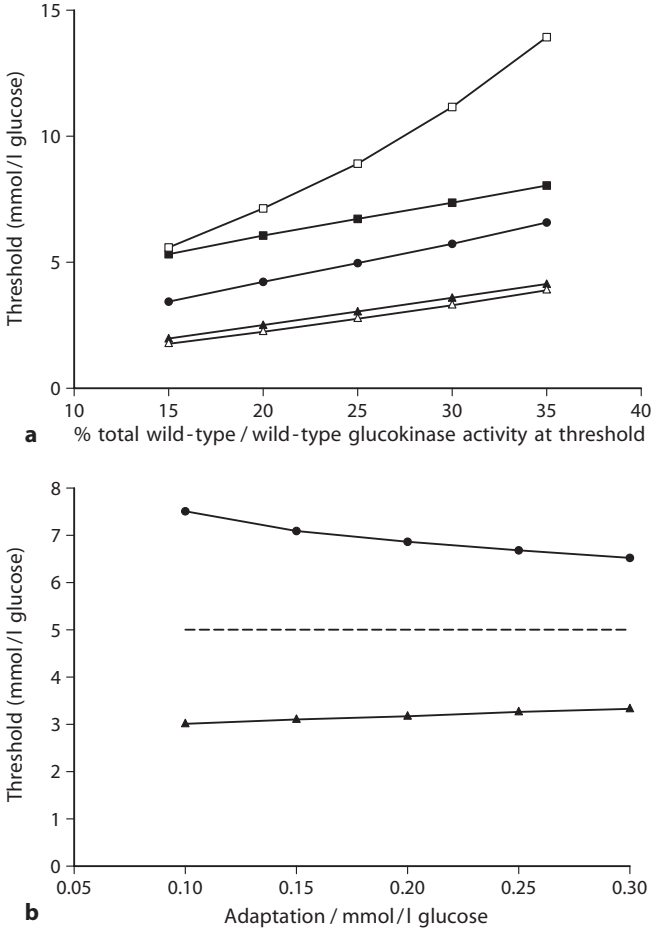

Fig. 4 a, b. Impact of assumptions used for mathematical modelling. **a** Using the known kinetics for wild-type and 2 of the mutants (C213R is a DM-GK mutant and V455M is a hypoglycaemia mutant) the assumed per cent of total phosphorylating capacity in the normal wild-type β-cell needed for insulin secretion was varied from 15% to 35% (25.4%, the relative rate at 5 mmol/l glucose is used in the modelling calculations presented in Figure 3). (—●— Wt; —□— C213R, not adapted; —■— C213R, adapted; —△—V455M, not adapted; —▲— V455M, adapted. **b** The effect of adaptation to increased glucose on GSIR threshold was studied over a threefold range. A factor of 0.2 was observed experimentally [29] and was used in the modelling presented in Fig. 3. (– – – – Wt; —●— C213R, —▲— V455M

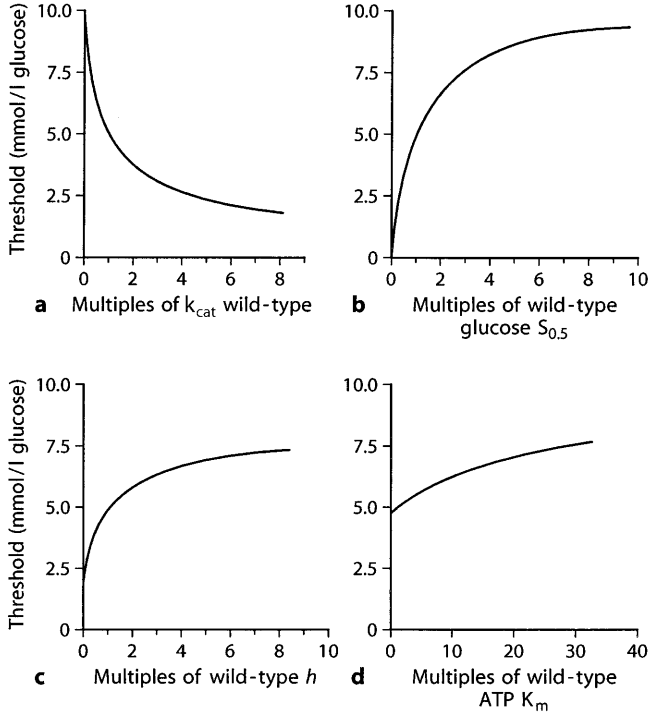

Fig. 5 a–d. Effect of changing one kinetic variable at a time on beta-cell threshold for GSIR. The beta-cell threshold for GSIR was modelled as a function of changing one glucokinase kinetic variable (k_{cat}, glucose $S_{0.5}$, h and ATP K_m) at a time. Thresholds were calculated for each variable approaching but not equal to zero

glucokinase activity is by a factor of 0.2 per mmol/l glucose. Over a range of 0.1–0.3 per mmol/l glucose the effect on glucose threshold is minimal.

Many of the mutants have more than one kinetic difference from the wild-type. Some are different in every aspect of glucokinase biochemistry. To tease out which kinetic changes have the greatest impact on threshold, we modelled a number of theoretical mutants. One kinetic variable at a time, k_{cat}, glucose $S_{0.5}$ and h and the ATP K_m was changed over a range of values and the predicted non-adapted threshold determined (Figure 5a-5d). The k_{cat} of the mutant allele had a profound effect on the glucose threshold. Once the k_{cat} of the mutant allele falls to less than

Table 3. Testing the model with clinical data

	Fasting blood glucose, mmol/l		
	Controls	DM-gk	HI-gk
Mathematical Model	5 (by definition)	6.7 ± 0.1[b] (predicted)	3.09 (predicted)
G. Velho et al. [15]	5.0 ± 0.5[a] (n = 341)	7.0 ± 1.1[a] (n = 260)	–
R. Page et al. [31]	4.9 ± 0.4[a] (n = 12)	7.3 ± 1.8[a] (n = 17)	–
M. Byrne et al. [16]	5.0 ± 0.02[b] (n = 6)	6.7 ± 0.1[b] (n = 6)	–
F. Shimada et al. [32]	5.1 ± 0.2[b] (n = 8)	6.8 ± 0.4[b] (n = 5)	–
B. Glaser et al. [3]	–	–	2.25 (n = 2)

[a] means ± SD
[b] means ± SEM

5% of the wild-type k_{cat} its contribution to phosphorylation is minimal and the threshold approaches 10 mmol/l. Simply doubling the mutant k_{cat} lowers the threshold to hypoglycaemic concentrations of about 3 mmol/l glucose. If the glucose $S_{0.5}$ is increased to 20 mmol/l the glucose threshold reaches 7.5 mmol/l and any further increase in glucose $S_{0.5}$ will have relatively little additional impact, i.e. the threshold plateaus at 10 mmol/l (Fig. 5). Theoretically a mutant with a selected change in *h* could change the threshold in either direction. The Hill number would need to fall to 1.0 or increase to above 6 to make much of an impact on the threshold. Three mutations with statistically significant changes in the Hill numbers have been found to date (G80A, T168P and S336L) (Table 1) but other predominant kinetic changes determine the end result in these cases. Lowering the ATP K_m has only a marginal effect on the threshold because the enzyme is physiologically saturated 88% at 2.5 mmol/l beta-cell ATP. With increasing ATP K_m of glucokinase similar to those observed in some of the mutants the impact on the glucose threshold could, however, be very large. Figures 5a–5d illustrate that the relative control power of the four critical kinetic variables of glucokinase differs considerably depending on the magnitude and the direction of the mutational change. Future modelling will have to determine more precisely the combinatorial rules that govern the impact of multiple changes.

Discussion

There is a surprising variation of kinetic data for many of the DM-gk mutants and even wild-type glucokinase [15, 18–22, 24, 25]. Some is probably due to reporting of kinetic data for wild-type and mutant enzymes which inadvertently included the D158A amino acid substitution. It is also partly due to different assay conditions changing apparent behaviour of glucokinase, different purification procedures and techniques for kinetic analysis. The attention given to technical details in this report may seem excessive but if the ever increasing numbers of glucokinase gene mutations are to be analysed kinetically and contribute usefully to further knowledge about glucokinase structure-function relations and their role in glucose homeostasis, rigorous standards need to be met. Assay pH changes the glucose $S_{0.5}$ and lowering from pH of 8 to 6.8 can double the glucose $S_{0.5}$ with the most pronounced effect occuring below pH of 7.2 ([34], unpublished observations, Matschinsky et al.). We chose 7.4 because it is close to physiological pH and yet above pH of 7.2 where even small errors have a large impact on kinetics. Note also that more alkaline pH appears to decrease the efficacy of GKRP inhibition [18, 35] suggesting pH is critical for ligand induced modification of glucokinase activity. The temperature of the assay is critical as some of the mutants are unstable at higher temperatures. The assay was run at 30 °C and results converted to 37 °C [36]. Relative concentrations of Mg^{++} to ATP are very important as they change the amount of ATP in the form of $MgATP^{2-}$ which is the substrate for glucokinase [34]. Assays were therefore run with Mg^{++} 1 mmol/l in excess of ATP. Non-linear kinetic analysis accounting for glucokinase cooperativity provides a more exact kinetic profile than the various linearizing plots of the Michaelis-Menten equation [17, 22, 34]. At a minimum the kinetic analysis of a new mutant needs to include glucose $S_{0.5}$, k_{cat}, *h*, ATP K_m, effects of inhibitors, thermal stability and affinity for different hexoses (e.g. glucose, mannose and fructose) particularly in cases that show normal kinetics.

We have used these evolving guidelines in re-analysing nine mutants previously studied in this laboratory and in others. These new data are critical for a number of reasons. Improved purification techniques give better yields. Methods of ATP kinetics have been refined acknowledging the contribution both the substrates glucose and ATP have on the activity of the enzyme and using an equivalent glucose concentration at the $S_{0.5}$ rather than at an arbitrary level of 100 mmol/l or at saturation as done previously. At an intracellular ATP concentration of 2.5 mmol/l increasing the ATP K_m only a few fold has statistically significant effects and decreases overall glucokinase phosphorylation ability.

We studied D158A in this series because in most if not all earlier studies from other laboratories this particular mutation (usually assumed to represent the wild-type enzyme) was incidentally incorporated into the wild-type plasmid from which other mutants were derived [19–21, 33]. The glucose $S_{0.5}$ for this mutation is 3.7 mmol/l compared with 8.3 for wild-type (Table 1). In hindsight, this often explains the lower glucose $S_{0.5}$ frequently found in the literature for wild-type of 4–6 mmol/l [19–21, 33]. The use of the GST fusion system has been validated in our hands by obtaining the same kinetic data with pure glucokinase as the GST-GK form. These kinetic data are similar to those [37] using an alternative purification technique to purify glucokinase.

The difference in k_{cat} between the two protocols for some enzymes found in our study, raises the question of glucose-dependent enzyme stability. Perhaps under more ideal conditions even greater activity than we report here could be obtained for some of the mutants. For example E300K is one for which kinetic data in the literature vary strikingly. It is now known from in vitro [22] and cell biological in vivo [30] studies that this DM-gk mutant causes instability and hence even small differences in the purification or assay technique would result in different kinetic

findings. It is difficult to draw correlation between in vitro assays and what happens in the islet but the study of these mutants in a cellular system is essential for fully characterized glucokinase mutants.

Differences in $S_{0.5}$ of ± 50 % (or 4 mmol/l) between mutants and wild-type might be considered as non-significant but in the physiological range these small differences can make a big impact on GSIR and glucose homeostasis of the subject. A good example is V455M where the decrease in $S_{0.5}$ from 8.3 to 3.2 mmol/l is sufficient to cause severe hypoglycaemic symptoms in the subject including seizures.

We have found a wide range in the ATP K_m values of more than 100-fold from about 0.1 to about 22 mmol/l ATP in the mutations studied. This contrasts with previous data of a much narrower range for the ATP K_m of 0.1–0.2 mmol/l [25]. This greater range of the present and a previous study from this laboratory probably comes from recognizing the complex interplay between ATP and glucose, such as the change in ATP K_m with a change in glucose. Impact of mutants on ATP binding could be a critical factor for expanding the available structural model of glucokinase [38].

Three mutants, (H137R, E300K, V367M) have pronounced thermolability but normal glucose and ATP kinetics. It is notable that when these mutations were first identified, it was predicted from the model [38] that H137R and V367M affected residues far from the active site and would have little effect on catalysis [15]. The same was predicted for A53S and S336L. Our study finds kinetics similar to wild type for three of these, consistent with the prediction, but changed k_{cat}, *h*, ATP K_m values for S336L are vastly different from the expected. This new kinetic data could be useful to further predict the structure and behaviour of the glucokinase molecule and its substrate binding site(s) in particular on ATP binding [39].

Certain mutants stand out as needing further evaluation. The cooperative nature of glucokinase is not understood. Mutants T168P and S336L which have an *h* of close to 1 and retain adequate activity to allow reliable assays are good candidates for a detailed study aiming to shed light on the mechanisms of cooperativity. Most mutants with loss of cooperativity have high ATP K_m which suggests a possible role of ATP binding in cooperative behaviour. The G261R and S336L mutants have very high ATP K_m but retain appreciable activity which makes them suitable for systematic studies of ATP binding using site directed mutagenesis. The one mutant of the 20 studied that stands out as having no kinetic cause for abnormal phenotype was A53S. This mutation was described in one case and only the proband was available for clinical studies [15]. It is not known if there is an alternative cause for high blood glucose in this subject which might relegate the A53S change to a biologically silent polymorphism. Studies of this mutant enzyme in a cell biological test system could contribute to resolving the puzzle.

Because of the high control strength of glucokinase over beta-cell glucose metabolism it is possible to predict the impact of a change in the rate of glucose phosphorylation on the threshold for insulin secretion. The post-absorptive glucose threshold for GSIR can be calculated with the model as described here. Using the non-adapted formulation the threshold for any of the mutants, even those with total loss of activity, is no greater than 10 mmol/l. Hence, the conclusion is that one wild-type allele is capable of initiating insulin secretion once glucose reaches 10 mmol/l, or that at 10 mmol/l glucose the phosphorylating capacity of the beta-cell can reach 25 % of the capacity of beta-cell glucose phosphorylation. The contribution that the mutant glucokinase makes to phosphorylation is appreciable in some cases but not in others. In the latter cases the mutant lies to the right of the threshold curve in Figure 3 a.

The figure of adaptation or increased glucokinase activity with persistently increased glucose shows the small range the body is able to maintain glucose concentrations within, even with one non- functional or only partly functional glucokinase allele. The ability of one adapted wild-type allele to compensate for the loss of phosphorylation capacity is the combined result of an increased amount of enzyme and its normal kinetic features including its hysteretic nature with a Hill coefficient *h* of 1.8 and an $S_{0.5}$ of 8.3 mmol/l glucose, so that the generation of metabolic coupling factors and ultimately insulin secretion remains effective even though the enzyme is subnormal. The control strength of even one normal allele is such that heterozygotes with two severe glucokinase mutations can be predicted to have very high blood glucose and most could not be compatible with life. Hypothetical homozygotes or compound heterozygotes with very mild mutations would, however, survive and present as diabetic subjects. We predict such instances will be found.

The hypoglycaemic mutant V455M is slightly different in adaptation. With a threshold of only 2.25 mmol/l, glucokinase activity of the wild-type allele can be expected to decrease with adaptation curbing hyperinsulinaemia. In reality, the beta-cells of these patients probably are exposed to higher average concentrations than threshold glucose because in order to minimize hypoglycaemic symptoms these patients learn to eat small and frequent meals and so in vivo adaptation is possibly less than we have adjusted for in the model. This could also explain the discrepancy between predicted and actual threshold (Table 3).

Modelling of in vitro data is illuminating conceptually but the critical test of the modelling results must make use of the relevant clinical data base. For this we have gone to the clinical studies from different

countries reported on some of the DM-gk families. Table 3 shows fasting blood glucose for over 250 DM-GK patients and reported values very close to the 6.7 mmol/l predicted by our model with adaptation. One of the families reported [16] had the G261R mutation we have studied here and can be identified on Figure 3a. Fasting glucose reported was 6.8 mmol/l close to the predicted 6.9 mmol/l on Figure 3b. Glucose infusion studies extending for 48 h showed increased glucose responsiveness of beta-cells consistent with the concept of adaptation of the wild-type allele. A model for some of the clinical data has been presented to predict the consequences on insulin secretion with glucokinase mutations [23] but it used calculations which did not include the sigmoidicity of glucokinase, neglected the role of the second substrate ATP and because of inclusion of the D158A double mutant in the data base used for G261R kinetics the association between the clinical and kinetic data were not as close as we have found here. With improved understanding of the relation between glucose phosphorylation and insulin secretion it is now clear the assumption that the more 'severe' the kinetic effect of a glucokinase mutation the more severe the phenotype is not as straight forward as once thought. As can be seen in the curves in Figure 5 when the glucose $S_{0.5}$ is greater than 20 mmol/l any increase has little further effect on threshold. So a mutant with a glucose $S_{0.5}$ of 150 mmol/l although it is undoubtedly more severe than a mutant with $S_{0.5}$ of 20 mmol/l has practically the same impact on glucose threshold in the beta-cell and the clinical picture would be expected to be the same. Instability of a kinetically normal DM-gk mutant makes extrapolation even more difficult.

Although DM-gk in itself is not a disease with serious consequences, the link between glucokinase mutations and changed glucose homeostasis in both DM-gk and HI-gk provide an opportunity to further illuminate the mechanisms of glucose sensing and insulin secretion in the beta-cell. Figures 3a and 3b which present the beta-cell threshold for GSIR related to activity of islet glucokinase and β-GPR is critical to the understanding of glucose homeostasis in the normal person and provides insight into how the mutations of glucokinase change the threshold for insulin secretion in the pathological conditions of both excess and deficient insulin secretion. Clearly neuroendocrine factors involving the central and peripheral nervous system could modify this glucokinase control of GSIR but in the narrow range of 3 to 7 mmol/l glucose, bracketed by DM-gk and HI-gk-based syndromes, glucokinase is the predominant factor. Small relative changes of its concentration or function have considerable consequences on glucose homeostasis very similar to the impact haemoglobin mutations have on O_2-homeostasis. Glucokinase and haemoglobin are molecules with very high control strength in the regulation of blood glucose and O_2, respectively.

The great challenge remains to evaluate the close to 100 known mutations causing DM-gk. The careful comprehensive characterization of glucokinase mutants may provide further clues about the basic biochemistry and biophysics of the enzyme and about the beta-cell's link between glucose metabolism and insulin secretion. One of the ways to tackle this will be to test the mutants in a cellular system where factors not yet recognized perhaps play an important regulatory role and another will be to develop new pharmacological agents that might activate both wild-type and mutant glucokinase. We have presented a model for predicting the threshold for GSIR dependent upon glucose phosphorylation in the beta-cell. This model could be taken further to predict the impact of changed glucokinase phosphorylation (by either glucokinase mutation or modifying agents) on postprandial insulin secretion. This expanded model might incorporate approaches of an earlier modelling which allowed prediction of the rate of insulin secretion over wide ranges of beta-cell glucokinase activities and ambient glucose concentrations [40]. Clearly factors such as insulin resistance, GLP-1 and parasympathetic stimulation make this a more complex, but nonetheless an interesting question.

The mathematical model we have developed to link beta-cell phosphorylation and insulin release shows that an increase in insulin secretion and lowering of GSIR threshold is feasible by moderately increasing glucokinase activity pointing to the possibility that endogenous or pharmacological agents will be found which activate wild-type or mutant glucokinase.

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