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ARTICLE

Neonatal diabetes caused by a homozygous *KCNJ11* mutation demonstrates that tiny changes in ATP sensitivity markedly affect diabetes risk

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Abstract
Aims/hypothesis The pancreatic ATP-sensitive potassium (K_{ATP}) channel plays a pivotal role in linking beta cell metabolism to insulin secretion. Mutations in K_{ATP} channel genes can result in hypo- or hypersecretion of insulin, as in neonatal diabetes mellitus and congenital hyperinsulinism, respectively. To date, all patients affected by neonatal diabetes due to a mutation in the pore-forming subunit of the channel (Kir6.2, *KCNJ11*) are heterozygous for the mutation. Here, we report the first clinical case of neonatal diabetes caused by a homozygous *KCNJ11* mutation.
Methods A male patient was diagnosed with diabetes shortly after birth. At 5 months of age, genetic testing revealed he carried a homozygous *KCNJ11* mutation, G324R, (Kir6.2-G324R) and he was successfully transferred to sulfonylurea therapy (0.2 mg kg^{-1} day^{-1}). Neither heterozygous parent was affected. Functional properties of wild-type, heterozygous and homozygous mutant K_{ATP} channels were examined after heterologous expression in *Xenopus* oocytes.
Results Functional studies indicated that the Kir6.2-G324R mutation reduces the channel ATP sensitivity but that the difference in ATP inhibition between homozygous and heterozygous channels is remarkably small. Nevertheless, the homozygous patient developed neonatal diabetes, whereas the heterozygous parents were, and remain, unaffected. Kir6.2-G324R channels were fully shut by the sulfonylurea tolbutamide, which explains why the patient's diabetes was well controlled by sulfonylurea therapy.
Conclusions/interpretation The data demonstrate that tiny changes in K_{ATP} channel activity can alter beta cell electrical activity and insulin secretion sufficiently to cause diabetes. They also aid our understanding of how the Kir6.2-E23K variant predisposes to type 2 diabetes.

Keywords ATP-sensitive potassium channel · ATP sensitivity · *KCNJ11* · Neonatal diabetes · Type 2 diabetes

Abbreviations

DEND	Developmental delay, epilepsy and neonatal diabetes
Het	Heterozygous mutation, e.g. hetG324R
Hom	Homozygous mutation, e.g. homG324R
iDEND	Intermediate DEND
K_{ATP} channel	ATP-sensitive potassium channel
Kir	Potassium inward rectifier
PNDM	Permanent neonatal diabetes mellitus
SUR	Sulfonylurea receptor
TNDM	Transient neonatal diabetes mellitus
WT	Wild-type

Introduction

Neonatal diabetes is a rare genetic disorder characterised by diabetes that presents within the first 6 months of life and

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which may be either permanent (PNDM) or transient (TNDM). Approximately 50% of cases of neonatal diabetes are due to gain-of-function mutations in the genes that encode the pore-forming (*KCNJ11*, encoding the inwardly rectifying potassium channel Kir6.2) or regulatory (*ABCC8*, encoding the sulfonylurea receptor [SUR] 1) subunits of the ATP-sensitive potassium (K_{ATP}) channel [1–3]. In ~30% of these patients, neurological symptoms such as developmental delay and muscle hypotonia are also found, a condition termed intermediate DEND (iDEND) syndrome [2]. About 3% of patients also experience epilepsy (DEND syndrome, defined as developmental delay, epilepsy and neonatal diabetes). K_{ATP} channel mutations are also associated with diabetes that presents in later life [4–6], and a common polymorphism in *KCNJ11* (E23K) confers an enhanced risk of type 2 diabetes [7–9].

The K_{ATP} channel plays a fundamental role in multiple tissues by coupling cell metabolism to electrical activity and thereby cell function. In pancreatic beta cells, it links plasma glucose levels to insulin secretion, and in neurons it modulates neuronal activity and neurotransmitter release [10]. Metabolic regulation is mediated via changes in intracellular adenine nucleotide levels, with ATP closing the channel by binding to Kir6.2 and Mg nucleotides, stimulating channel opening by interaction with SUR1 [11–13]. In the beta cell, K_{ATP} channel closure leads to electrical activity, calcium influx and thereby insulin granule exocytosis. Activating K_{ATP} channel mutations cause neonatal diabetes by impairing channel inhibition by MgATP and thereby preventing glucose-induced insulin secretion [1–3, 10]. Compared with PNDM (or TNDM), DEND and iDEND syndromes are associated with functionally more severe mutations that cause a greater reduction in ATP sensitivity and thus affect neurons as well as beta cells [10]. It is believed that a very small reduction in ATP inhibition underlies the ability of the Kir6.2-E23K variant to enhance type 2 diabetes risk [14–16]. However, this has been difficult to prove conclusively.

To date, all patients with neonatal diabetes due to mutations in the gene encoding Kir6.2 have been heterozygous for the mutation. Identification of a homozygous mutation causing recessive neonatal diabetes, however, would clearly define the extent of the reduction in channel ATP sensitivity that is sufficient to cause neonatal diabetes and also help determine whether the small decrease in ATP sensitivity caused by the Kir6.2-E23K variant is enough to account for the increased type 2 diabetes risk.

Methods

Molecular genetics Genomic DNA was extracted from peripheral leucocytes using standard procedures. The coding regions and conserved splice sites of the *ABCC8* and *KCNJ11* genes were amplified by PCR and the resulting amplicons sequenced using the BigDye Terminator Cycle v3.1 Sequencing Kit (Applied Biosystems, Warrington, UK). The products were analysed on an ABI 3730 capillary sequencer (Applied Biosystems) and compared with the reference sequences (NM_000525.3 and NM_000352.3) using Mutation Surveyor version 3.24 software (SoftGenetics, State College, PA, USA).

Molecular biology and oocyte preparation We used human *KCNJ11* (GenBank NM000525, with E23 and I337 [except where stated]) and rat *Abcc8* (GenBank L40624). Site-directed mutagenesis of *KCNJ11* was performed using the QuikChange XL system (Stratagene, La Jolla, CA, USA). *KCNJ11* and *Abcc8* mRNAs were prepared using the mMESSAGE mMACHINE large-scale in vitro transcription kit (Ambion, Austin, TX, USA), as described previously [17]. Defolliculated *Xenopus laevis* oocytes were injected with 0.8 ng wild-type (WT) (or mutant) *KCNJ11* mRNA and 4 ng *Abcc8* mRNA, and incubated in Barth's solution at 18°C for 1–4 days. To simulate the heterozygous state of unaffected heterozygous carriers, we coinjected a 1:1 mixture of mutant and WT *KCNJ11*, together with *Abcc8*. The resulting channel population (referred to here as heterozygous [het]G324R) will contain a variable number of mutant subunits (between zero and four) in the Kir6.2 tetramer.

Electrophysiology Whole oocyte currents were recorded using a two-electrode voltage clamp (GeneClamp 500B; Molecular Devices, Sunnyvale, CA, USA), in response to 500 ms voltage steps of ±20 mV from a holding potential of -10 mV. Data were acquired at 4 kHz, after online filtering at 0.5 kHz, using a 1440A Digidata interface (Molecular Devices), computer controlled by pCLAMP 10 software (Molecular Devices). Oocytes were continuously perfused with (in mmol/l) 90 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$ and 5 HEPES (adjusted to pH 7.4 with KOH), supplemented with sodium azide (3 mmol/l) or tolbutamide (0.5 mmol/l, diluted from a 50 mmol/l stock solution in DMSO), as indicated.

Macroscopic currents were recorded at -60 mV from giant excised inside-out patches, using an Axopatch 200B amplifier (Molecular Devices), filtered at 1 kHz and sampled at 10 kHz with a Digidata 1322A A/D-D/A driven by pCLAMP 9 software (Molecular Devices). The pipette (extracellular) solution contained (in mmol/l) 140 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$ and 10 HEPES (adjusted to pH 7.4 with KOH). The bath (cytoplasmic) solution contained (in mmol/l) 107 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 10 HEPES (adjusted to pH 7.2 with KOH) and MgATP, as indicated.

MgATP concentration–response curves were individually fitted with the Hill equation: $I/I_C = 1/(1 + ([MgATP]/IC_{50})^h$, where [MgATP] indicates the MgATP concentration, IC_{50} is the MgATP concentration that causes half-maximal block, I_C

and I are the currents in the absence and presence of nucleotide, respectively, and h is the Hill coefficient. To correct for K_{ATP} current rundown, I_C was taken as the mean of the current in nucleotide-free solution before and after each ATP application. In addition, only patches in which the response to 100 μmol/l MgATP did not change during the course of the experiment were included in the analysis. Data were analysed with Clampfit (pCLAMP 9 or 10; Molecular Devices) and Origin 7.0 (OriginLab, Northampton, MA, USA). Results are given as mean ± SEM of *n* measurements from at least two independent batches of oocytes.

Results

Clinical and genetic data A male patient of Indian ethnicity was diagnosed with diabetes at 11 weeks of age (birthweight 2,700 g at term). At the age of 5 months, following a referral for genetic testing, sequence analysis identified a novel homozygous missense mutation, p.G324R (c.970G>A), in the *KCNJ11* gene (referred to here as Kir6.2-G324R). Sequence analysis of the *ABCC8* gene or insulin gene did not identify a mutation.

Following genetic diagnosis, the patient transferred from insulin (2.1 U kg^{-1} day^{-1}) to glibenclamide (0.2 mg kg^{-1} day^{-1}), with an improvement in HbA_{1c} (8.4% pre-transfer to 5.6% post-transfer [68 mmol/mol to 38 mmol/mol]). The patient is currently 4.5 years of age and is not requiring any treatment. The age at remission of diabetes is unknown.

Both parents, who are not diabetic at the age of 29 years and 34 years, respectively, were heterozygous for the Kir6.2-G324R mutation. Samples from the unaffected grandparents were not available for testing.

Metabolic regulation of the K_{ATP} channel We analysed the effects of the Kir6.2-G324R mutation on metabolic regulation of the K_{ATP} channel by measuring whole-cell currents. When WT K_{ATP} channels are expressed in *Xenopus* oocytes they are normally closed, due to the high intracellular ATP concentration. However, they can be opened by lowering the intracellular ATP concentration using a metabolic inhibitor such as sodium azide (Fig. 1a). Mutations that reduce the channel ATP sensitivity normally increase the whole-cell current in control solution, reflecting the fact that they are less blocked by the resting intracellular ATP concentration [10, 18]. Homozygous Kir6.2-G324R/SUR1 currents (homG324R) were not appreciably different from WT either in control solution or in the presence of azide (Fig. 1a).

To control for variability between different oocytes (and different batches of oocytes), we expressed the resting current in control solution as a percentage of the maximal current recorded in the presence of 3 mmol/l sodium azide. Mean data are given in Fig. 1b. There was no difference between WT and heterozygous Kir6.2-G324R/SUR1 currents (hetG324R),

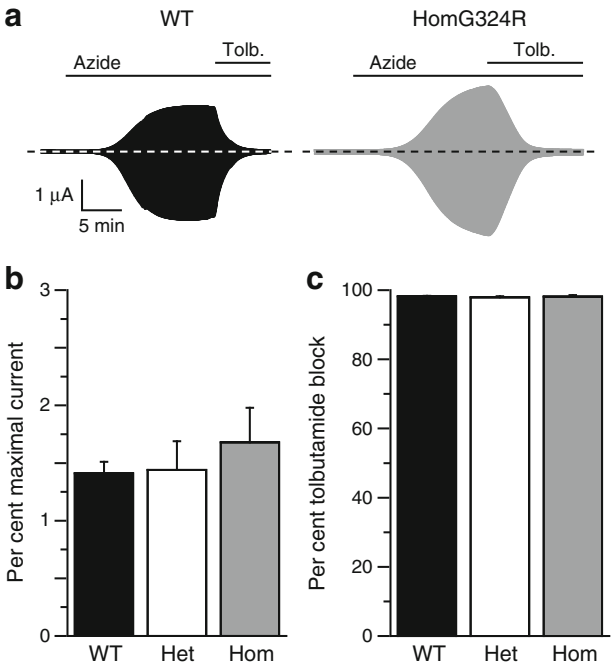

Fig. 1 Metabolic activation of WT and mutant K_{ATP} channels. (**a**) Representative whole-cell currents recorded from oocytes expressing WT or homG324R channels in response to 500 ms voltage steps of ±20 mV from a holding potential of −10 mV. Sodium azide (azide, 3 mmol/l) and tolbutamide (tolb., 0.5 mmol/l) were added as indicated. (**b**) Current in control solution for WT ($n = 13$), hetG324R (het; $n = 9$) and homG324R (hom; $n = 10$) expressed as a percentage of that in the presence of 3 mmol/l sodium azide (per cent maximal current). (**c**) Mean tolbutamide block for WT (98.2 ± 0.2%, $n = 7$), hetG324R (het; 97.9 ± 0.3%, $n = 6$) and homG324R (hom; 98.1 ± 0.4%, $n = 7$) channels (measured in the presence of sodium azide)

which may explain why heterozygous carriers did not develop neonatal diabetes. HomG324R currents were slightly greater than either WT or hetG324R currents, although this difference did not reach significance.

The K_{ATP} channel inhibitor tolbutamide (0.5 mmol/l) blocked all three types of channel by ~98% (Fig. 1c). This explains why the neonatal diabetes of homG324R patients can be controlled by sulfonylurea therapy. Indeed, homG324R channels were blocked by tolbutamide as efficiently as WT channels, which suggests the patient may be at somewhat higher risk of hypoglycaemia than patients with more strongly activating K_{ATP} channel mutations that are less sensitive to sulfonylureas.

ATP sensitivity We examined the ATP sensitivity of WT and mutant channels in the presence of 2 mmol/l Mg^{2+}, to approximate the physiological condition (Figs 2, 3). WT channels were half maximally blocked (IC_{50}) by 18 ± 2 μmol/l MgATP ($n = 13$). The ATP sensitivity of homG324R channels was slightly but significantly smaller than WT ($IC_{50} = 38$ ± 3 μmol/l, $n = 8$; $p < 0.01$ vs WT) (Figs 2b, 3a). The ATP sensitivity of hetG324R channels was also reduced but the difference was not significant ($IC_{50} = 30 \pm 3$ μmol/l, $n = 6$

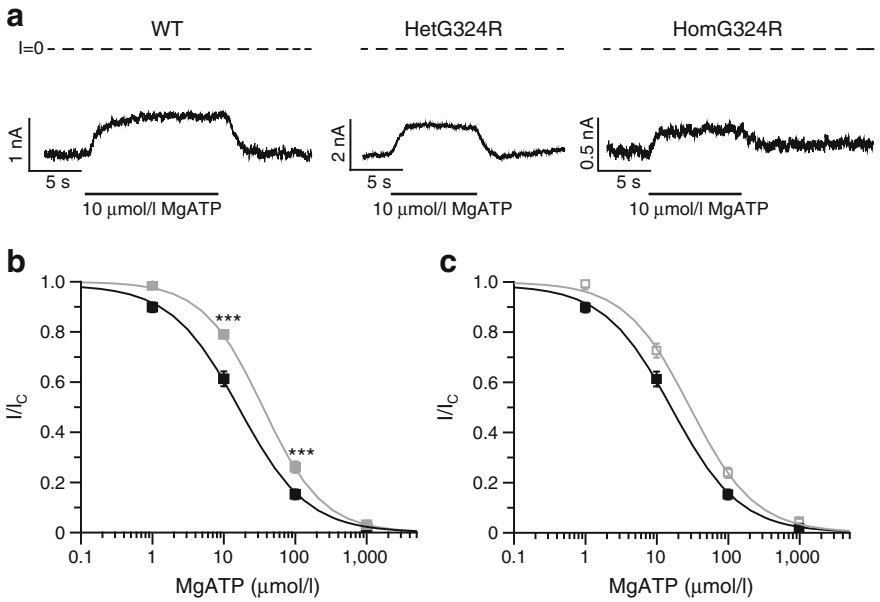

Fig. 2 MgATP inhibition of K_{ATP} channels is reduced by the Kir6.2-G324R mutation. (**a**) Representative currents recorded at −60 mV from inside-out patches excised from oocytes expressing WT or mutant K_{ATP} channels. The dashed line indicates the zero current level. MgATP (10 μmol/l) was applied as indicated. (**b**, **c**) Relationship between K_{ATP} current and MgATP concentration for WT (black squares), homG324R (grey filled squares; **b**) and hetG324R (grey empty squares; **c**) channels. Current in the presence of nucleotide (I) is expressed as a fraction of that in its absence (I_C). The curves are the best fit to the Hill equation. ***$p < 0.001$ compared with WT (*t* test)

(Figs 2c, 3a). For comparison, the IC_{50} was 21 ± 2 μmol/l ($n = 5$) for K_{ATP} channels containing the Kir6.2-K23 variant.

The K_{ATP} current amplitude at physiological ATP concentrations is critical, as this will determine the beta cell resting potential and thus the clinical phenotype. At 3 mmol/l MgATP, a concentration within the physiological range, the percentage of unblocked current was 1.1 ± 0.2% ($n = 8$) for homG324R channels and 1.2 ± 0.3% ($n = 6$) for hetG324R channels, compared with 0.8 ± 0.2% ($n = 13$) for WT channels. These differences were not significant (Fig. 3b).

We observed no significant difference in homG324R and WT current magnitudes in control solution in excised patches, suggesting the Kir6.2-G324R mutation does not alter membrane trafficking.

Discussion

The difference in ATP sensitivity between recombinant K_{ATP} channels homomeric for the Kir6.2-G324R mutation and

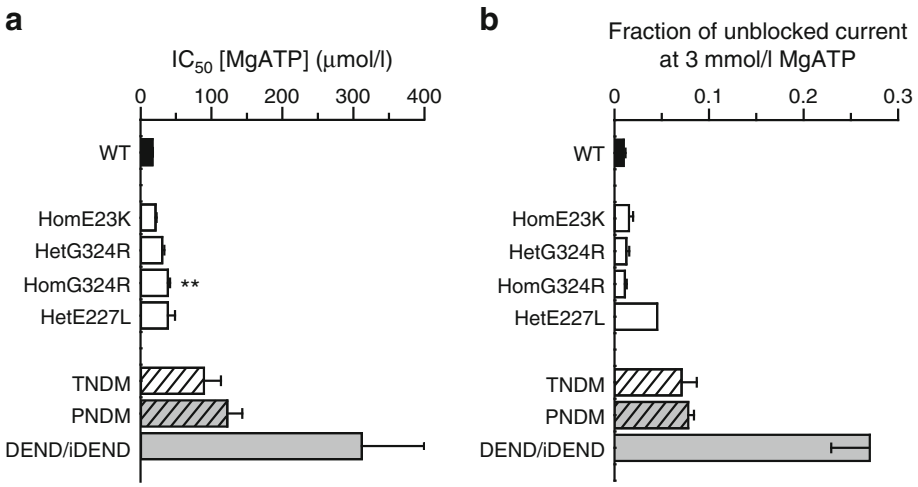

Fig. 3 Comparison of ATP sensitivities. (**a**) IC_{50} for MgATP inhibition and (**b**) fraction of unblocked current at 3 mmol/l MgATP for the indicated channels: WT ($n = 13$), homE23K ($n = 5$), hetG324R ($n = 6$), homG324R ($n = 8$), hetE227L ($n = 7$). Mean values for TNDM (white hatched bars), PNDM (grey hatched bars) and DEND/iDEND (grey bars) channels are given for comparison (for references, see ESM Fig. 1). Data are mean ± SEM. **$p < 0.01$ compared with WT (*t* test)

pseudo-heterozygous hetG324R channels is strikingly small: the IC_{50} differs by only 8 μmol/l; yet, the proband homozygous for the G324R mutation developed neonatal diabetes, whereas his heterozygous parents were unaffected. Thus, tiny changes in ATP sensitivity can cause disease. This is not surprising, however, given that very small differences in K_{ATP} current magnitude can lead to marked differences in beta cell membrane potential and electrical activity (and thus insulin secretion), if they occur close to the action potential threshold, where the electrical resistance of the beta cell membrane is very high [19]. This is because the K_{ATP} current dominates the beta cell resting membrane potential. For a detailed explanation of how the beta cell membrane potential (and electrical activity) can be exquisitely sensitive to tiny changes in K_{ATP} channel activity, see the review by Ashcroft and Rorsman [20].

The ATP sensitivity measured for homG324R channels (38 μmol/l) lies within the lower range of that reported for heterozygous *KCNJ11* mutations associated with PNDM (range 34–273 μmol/l) or TNDM (range 27–213 μmol/l) (electronic supplementary material [ESM] Fig. 1). For comparison, the mean IC_{50} value obtained for DEND/iDEND mutations is 312 ± 89 μmol/l (n = 11), for PNDM it is 122 ± 21 μmol/l (n = 10), for TNDM it is 89 ± 22 μmol/l (n = 8) and for WT channels it is ~15 μmol/l (Fig. 3a). Thus, the more severe clinical phenotype (DEND/iDEND) is associated with a greater reduction in ATP sensitivity. Interestingly, the Kir6.2-E227L mutation that causes a variable disease phenotype, where some patients do not develop diabetes until early adult life or only during pregnancy, causes a much smaller reduction in ATP sensitivity [5], comparable with that observed for the homG324R mutation (Fig. 3a). Similarly, the percentage of unblocked current at 3 mmol/l MgATP was greater for mutations associated with a more severe clinical phenotype (Fig. 4b, ESM Fig. 1b).

Mechanism of action In a molecular model of Kir6.2 (Fig. 4), G324 lies far from the putative ATP-binding site [21] and thus its mutation is unlikely to impair ATP binding directly. Rather, it is expected to reduce ATP inhibition indirectly, either by allosterically impairing ATP binding or by enhancing the intrinsic open probability of the channel [18]. Importantly, the adjacent residue, D323, forms an ion pair with K1322 in SUR2A [22], and may be predicted to form a similar interaction with the equivalent residue in SUR1 (K1355). Furthermore, mutation of D323 to the positively charged amino acid lysine, which would disrupt the Kir6.2–SUR1 pair, causes PNDM, reducing the channel ATP sensitivity to 129 μmol/l in the heterozygous state and to 408 μmol/l in the homozygous state [23]. Potentially, therefore, the G324R mutation, which also introduces a positive charge, might disrupt the adjacent Kir6.2–SUR1 ion pair.

Relevance to type 2 diabetes A common polymorphism (E23K) in *KCNJ11* is associated with increased susceptibility to type 2 diabetes [8]. Although the increase in risk is relatively small (OR 1.18), it is highly significant. Functional studies have shown that in people with normal glucose tolerance, the K variant is associated with a marked (40%) reduction in insulin secretion in response to an oral or intravenous glucose challenge [9].

The molecular basis of the increased predisposition to type 2 diabetes remains unclear, as the reported effect of the mutation on K_{ATP} channel activity differs between different laboratories. Most studies have found the K variant is associated with a very small reduction in sensitivity to ATP inhibition [9, 14]. However, others have reported that it enhances ATP sensitivity [24] or is without effect [16]. Similarly, increased activation by MgADP of Kir6.2-K23 channels was observed in one study [25], but not found in another [16]. Enhanced activation of Kir6.2-K23 channels by long-chain acyl-CoAs has also been observed [24]. A further complication is that the E23K polymorphism is linked both to a second polymorphism in *KCNJ11* (I337V) and to a polymorphism in *ABCC8* (A1369S) [26], and a recent study suggested that it was the SUR1-A1369S variant (not the Kir6.2-E23K polymorphism) that was the critical determinant of the difference in K_{ATP} channel ATP sensitivity [16]. This may be attributable to the

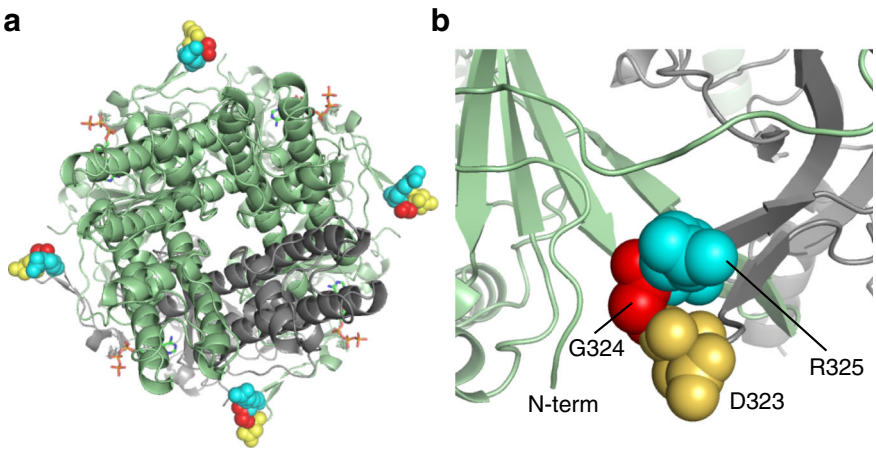

Fig. 4 Molecular model of the Kir6.2 tetramer [19]. (**a**) Top view from the extracellular side, showing the position of G324 (red), D323 (yellow) and R325 (cyan). ATP (orange sticks) is shown docked into its putative binding site. (**b**) Detail of two adjacent Kir6.2 subunits (one green, one grey). The first 32 amino acids are not included in the model so N-term denotes residue 32

increased ability of MgATP (via its hydrolysis) to activate the SUR1-A1369 variant [27].

Our finding that a tiny difference in ATP sensitivity—from 30 μmol/l (hetG324R) to 38 μmol/l (homG324R)—is sufficient to cause neonatal diabetes may help to resolve this conundrum, as it predicts an even smaller reduction in ATP sensitivity might be sufficient to predispose to type 2 diabetes. Given the usual biological variability, such a small difference may be hard to detect above the background noise. It is worth noting that genetic studies required >1,000 patients to detect a significant increase in risk [8]—a sample size not possible in electrophysiological experiments. In addition, differences in cell type, the amount of phosphatidylinositol 4,5-bisphosphate (PIP_2) in the membrane, and methodology (such as the time after patch excision that ATP sensitivity is measured) may contribute to the small differences seen between laboratories.

Our data help explain why patients with the Kir6.2-E23K polymorphism do not develop diabetes at birth: the reduction in ATP sensitivity is simply too small. They also clarify why it has been so difficult to detect differences in K_{ATP} channel function due to the Kir6.2-E23K/SUR1-A1369S mutations, as they reveal that the difference in ATP sensitivity between mutations causing neonatal diabetes (38 μmol/l) or not (30 μmol/l) is extremely small. Finally, they suggest the unaffected heterozygous Kir6.2-G324R parents may be at increased risk of developing type 2 diabetes in later life. Whether they do so may depend on their genetic background and environmental factors such as an obesogenic lifestyle.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement NV and FMA designed the study. NV collected and analysed both patch-clamp and two-electrode voltage clamp data, EC collected and analysed two-electrode voltage clamp data, and PP collected and analysed patch-clamp data. VP, SE, SEF and ATH collected the clinical data and identified the mutation. FMA, NV and SEF wrote the manuscript. All authors contributed to revising the article critically for important intellectual content and gave their final approval of the version to be published. FMA is the guarantor of the electrophysiological studies, and SE the guarantor of the genetic studies.

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