A rare mutation in ABCC8/SUR1 leading to altered KATP channel activity and β-cell glucose sensing is associated with type 2 diabetes mellitus in adults

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ABSTRACT

Objective: ATP-sensitive K⁺ (K<sub>ATP</sub>) channels link glucose metabolism to the electrical activity of the pancreatic β-cell to regulate insulin secretion. Mutations in either the Kir6.2 or SUR1 subunit of the channel have previously been shown to cause neonatal diabetes. We describe here an activating mutation in the ABCC8 gene, encoding SUR1, that is associated with the development of type 2 diabetes mellitus only in adults.

Research Design and Methods: Recombinant K<sub>ATP</sub> channel subunits were expressed using pIRES2-based vectors in HEK293 or INS1(832/13) cells and the subcellular distribution of c-myc-tagged SUR1 channels analysed by confocal microscopy. K<sub>ATP</sub> channel activity was measured in inside-out patches, and plasma membrane potential in perforated whole-cell patches. Cytoplasmic [Ca<sup>2+</sup>]<sub>cyt</sub> was imaged using Fura-red.

Results: A mutation in ABCC8/SUR1, leading to a Y356C substitution in the seventh membrane-spanning α-helix, was observed in a patient diagnosed with hyperglycemia at age 39, and in two adult offspring with impaired insulin secretion. Single K<sub>ATP</sub> channels incorporating Y356C-SUR1 displayed lower sensitivity to MgATP (IC₅₀=24µmol/l and 95µmol/l for wild-type and mutant channels, respectively). Similar effects were observed in the absence of Mg<sup>2+</sup>, suggesting an allosteric effect via associated Kir6.2 channels. Over-expression of Y356C-SUR1 in INS1(832/13) cells impaired glucose-induced cell depolarisation and increases in intracellular free Ca<sup>2+</sup> concentration, albeit more weakly than neonatal diabetes-associated SUR1 mutants.

Conclusions: An ABCC8/SUR1 mutation with relatively minor effects on K<sub>ATP</sub> channel activity and β-cell glucose sensing causes diabetes in adulthood. These data suggest a close correlation between altered SUR1 properties and clinical phenotype. [249 words]

ABBREVIATIONS. [Ca<sup>2+</sup>]<sub>cyt</sub>, cytosolic free Ca<sup>2+</sup> concentration; KRB, Modified Krebs’ Ringer bicarbonate medium; PND, TND, permanent and transient neonatal diabetes mellitus, respectively; T2D, type 2 diabetes, wt, wild-type
Glucose and other nutrient secretagogues trigger insulin secretion from pancreatic β-cells, in large part through the metabolism-dependent closure of ATP-sensitive K⁺ (K\(_{\text{ATP}}\)) channels. This, in turn, leads to plasma membrane depolarisation, Ca\(^{2+}\) influx and the exocytosis of dense core secretory vesicles (1,2).

K\(_{\text{ATP}}\) channels exist as heterooctamers (3) comprising four pore-forming (Kir6.2) and four regulatory (SUR1) subunits (Fig.1A), encoded by KCNJ11 and ABCC8 genes respectively. Mutations in either gene that reduce the metabolic sensitivity of β-cell K⁺ conductance have been shown to cause transient or permanent neonatal diabetes mellitus (TND and PND, respectively) (4-8). In either case, inhibited β-cell stimulus-secretion coupling (9) leads to insulin secretory insufficiency (10). The extent of the shift in the sensitivity of the mutant channels to ATP appears to be correlated with the severity of the disease (6), though up to now this relationship has only been demonstrated for mutations in KCNJ11/Kir6.2. Moreover, a single-nucleotide polymorphism in KCNJ11 gene, E23K (11), is associated with type 2 diabetes (12) and reduces the metabolic sensitivity of K\(_{\text{ATP}}\) channels by reducing the inhibitory effect of ATP (13) and/or enhancing activation by free fatty acids (14). By contrast, ABCC8/SUR1 mutations leading to adult-onset type 2 diabetes (T2D), without antecedent remitting diabetes during infancy (TND) have not been described. However, a number of mutations in ABCC8 (as well as KCJN11) were identified in adults who had not yet developed diabetes (15). Whether these represent mild mutations which may lead to diabetes in later life, or are without effect on K\(_{\text{ATP}}\) channel properties, is presently unknown.

Here, we report three novel mutations in ABCC8 that are associated with relatively mild insulin secretory deficiencies or T2D in adult patients. Through electrophysiology and Ca\(^{2+}\) imaging we demonstrate that one of the mutations, Y356C, affects the ATP sensitivity of K\(_{\text{ATP}}\) channels and glucose-induced Ca\(^{2+}\) influx, but to a far smaller extent than TND-associated mutations. We also use the information obtained for this and other mutations, and molecular modeling, to provide new insights into the interaction between Kir6.2 and SUR1 within the K\(_{\text{ATP}}\) channel complex.

**RESEARCH DESIGN AND METHODS**

**Study population and gene screening.** 187 adult subjects diagnosed with type 2 diabetes or hyperglycemia before age of 40 years (all of French Caucasian origin, except one subject with Antilla-black ancestry) entered the study for gene screening. The 39 exons of the ABCC8 gene were sequenced from genomic DNA in the patients, as previously described (4).

**Molecular biology and expression of recombinant channels.** cDNA encoding mouse Kir6.2 (CoreNucleotide NM_010602) or hamster SUR1 (CoreNucleotide L40623) were subcloned into plasmids pcDNA3 and pIRE2, respectively. Nucleotide substitutions were introduced into SUR1 cDNA using Quick-Change site-directed mutagenesis kit (Stratagene). The primers used for the mutagenesis are given in Supplementary Table 1 (Located in the online appendix (available at http://dx.doi.org/10.2337/db07-1547). We used pIRE2-EGFP and/or pIRE2-dsRed2 (Clontech) vectors to allow channel-independent expression of reporter proteins, EGFP (mutant SUR1) and dsRed2 (wild-type SUR1).

HEK293 or INS1(832/13) (16) cells were plated (1×10^5 cells/35mm dish), cultured overnight and co-transfected with pcDNA3-Kir6.2 and pIRE2-SUR1 cDNA in 7:3 ratio (HEK293 cells) or pIRE2-SUR1 on its own (INS1(832/13) cells), using Lipofectamine2000 (Invitrogen). Cells were studied two days later.

**Electrophysiology.** Currents were recorded using an EPC9 patch-clamp amplifier controlled by Pulse acquisition software.
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(HEKA Elektronik, Lambrecht/Pfalz, Germany). Inside-out patches excised from the membrane of HEK293 cells were recorded in response to three-second voltage ramps from -110mV to +100mV (holding potential, 0mV, see inset for Fig.2A), filtered at 0.15kHz and digitised at 0.5kHz. If the level of channel expression was low, $K_{\text{ATP}}$ currents were recorded as single-channel events at constant holding potential of -60mV, filtered at 1kHz and digitised at 2kHz. To control for possible rundown, the conductance ($G_c$) was taken as the mean of that in nucleotide-free solution before and after the application of ATP. For each recording, ATP concentration-inhibition curves were fitted to the Hill equation: \[ \frac{G}{G_c} = \frac{1}{1+(\text{[ATP]}/IC_{50})^h} \]
where $IC_{50}$ is the concentration at which inhibition is half-maximal and $h$ is the Hill coefficient. Given ATP inhibition values are the means of the fitted parameters for individual patches.

The plasma membrane potential of INS1(832/13) $\beta$-cells was recorded in perforated-patch whole-cell configuration. The pipette tip was dipped into pipette solution, and then back-filled with the same solution containing 0.24mg/ml amphotericin B. Recordings were initiated after 30 min. exposure to substrate-free solutions at 37°C and the duration of exposure to each concentration of effector(s) was $\geq$2 min. The frequency of action potentials was measured from recording intervals $\geq$1 min, corresponding to different extracellular solutions. For firing cells, the membrane potential was determined as the baseline potential between the spikes. Cells that were not responsive to tolbutamide were excluded from analysis. Series resistance and cell capacitance were compensated automatically by the acquisition software. Experiments were carried out by periodically switching from current-clamp to voltage-clamp mode, thus obtaining pseudo-simultaneous recordings of cell membrane potential ($V_m$) and $K_{\text{ATP}}$ conductance ($G_{K\text{ATP}}$) (9). This controlled for the leaks of the patch and verified that the depolarisation (hyperpolarisation) of the membrane was linked to $K_{\text{ATP}}$ channel closure (opening). The current clamp protocol involved continuous recording, without electrical stimulation. In the voltage clamp, the membrane potential was held at -70mV and whole-cell currents were evoked by $\pm$10mV 0.5Hz pulses. Data were filtered at 0.2kHz and digitised at 0.5kHz.

For inside-out patch recordings, the pipette solution contained (mmol/l): 140 KCl, 10 HEPES (pH 7.2 with KOH), 1.1 MgCl₂, 2.6 CaCl₂. The intracellular (bath) solution contained (mmol/l): 107 KCl, 1 CaCl₂, 2 MgCl₂, 11 EGTA, 10 HEPES (pH 7.2 with KOH), plus MgATP as indicated. Mg²⁺-free intracellular solutions contained (mmol/l): 107 KCl, 1 CaCl₂, 0.5 EDTA, 11 EGTA, 10 HEPES (pH 7.2 with KOH) and ATP as indicated. pH was measured after ATP addition and readjusted if required. For perforated-patch experiments the pipette solution contained (mmol/l): 76 K₂SO₄, 10 NaCl, 10 KCl, 1 MgCl₂, 5 HEPES (pH 7.35 with KOH). No ATP was added. The bath solution contained (mmol/l): 137 NaCl, 5.6 KCl, 10 HEPES (pH 7.4 with NaOH), 2.6 CaCl₂, 1.1 MgCl₂. All experiments were conducted at 21-23°C and the bath solution was perfused continuously.

**Measurements of cytoplasmic free Ca²⁺ concentration ([Ca²⁺]_{cys}).** Cells were preloaded by 40 min. incubation with 2µmol/l Fura-Red acetoxy-methyl ester (Invitrogen) (17,18) dissolved in KRB solution comprising (mmol/l): 130 NaCl, 3.6 KCl, 0.5 NaH₂PO₄, 0.5 MgSO₄, 2.0 NaHCO₃, 3 glucose, 10 Heps (pH 7.4 with NaOH) and 1.5 CaCl₂ equilibrated with O₂/CO₂ (95:5, v/v) at 37°C. Changes in [Ca²⁺]_{cys} were monitored at 0.2Hz, using a Cell^RTM (Olympus) epifluorescence imaging system, based around an Olympus IX-81 inverted optics microscope fitted with a x40 oil immersion objective. Cells were continuously perfused in KRB solution at the glucose concentrations indicated. The “KCl” solution comprised KRB in which 50mmol/l NaCl was substituted by KCl. [Ca²⁺]_{cys} was expressed as the ratio of
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fluorescence intensity (λ<sub>em</sub>=597nm, λ<sub>ex</sub>=440nm) to that at λ<sub>ex</sub>=490nm, after subtraction of background fluorescence. All experiments were performed at 37°C. To account for the differences in [Ca<sup>2+</sup>]<sub>cyt</sub> between individual cells, the fluorescence ratios at 20nmol/l glucose, in each trace, were normalised to the ratios observed at 3nmol/l glucose. Cells were selected by expression of the reporter proteins.

**Immunocytochemistry.** Cells were transfected with pIRES2-SUR1-c-myc or pIRES2-SUR1-c-myc plus pcDNA3-Kir6.2. 48h post-transfection, cells were stained with mouse anti-c-myc antibody clone (9E10, Roche). Cells transfected with SUR1 were fixed with 4% paraformaldehyde and permeabilised (methanol/acetone for HEK293 cells, Triton X-100 for INS1(832/13) cells) whereas cells transfected with SUR1 and Kir6.2 were fixed and directly stained for surface expression. After 2h incubation with primary antibodies cells were washed and labelled with goat anti-mouse Alexa 586 for 60 min., mounted using Prolong Gold antifade mounting media (Invitrogen) and observed with a Zeiss LSM510 confocal microscope.

**Data analysis and statistics.** Data was analysed using Clampfit (Axon Instruments), Cell^R (Olympus) and Excel (Microsoft) software. Unless specified otherwise (Fig.4D), statistical significance was estimated using Mann-Whitney U-test or Student’s t-test with Bonferroni correction for multiple sampling. Differences with P<0.05 were considered statistically significant.

**RESULTS**

**Identification of ABCC8 mutants and clinical data.** We screened for mutations in the ABCC8 gene in 204 diabetic subjects with disease onset before 40 years. One of the patients with normal BMI, diagnosed with hyperglycemia at age 39, having developed overt diabetes at 45, presented an ABCC8 missense mutation causing a substitution of tyrosine 356 with cysteine (Y356C) in the SUR1 subunit of the K<sub>ATP</sub> channel (Fig.1B, Supplementary Fig.1) (available at http://dx.doi.org/10.2337/db07-1547). The mutation was also identified in two children of the patient, aged 33 and 35, who showed normal fasting blood glucose levels but displayed a mild decrease of insulin secretion during an oral glucose tolerance test (Table 1, and Supplementary Information) (available at http://dx.doi.org/10.2337/db07-1547). The disposition index, as a measure of β-cell function related to insulin sensitivity status, was low in the two children, although they were not diagnosed with diabetes. The Y356C mutation was not found in 170 unrelated normoglycaemic individuals of European Caucasian origin.

The two other ABCC8 mutations that we found to be associated with adult onset diabetes were: R248Q (T2D patient diagnosed at 39 years without familial cosegregation, Supplementary Fig.1) and K1521N (two T2D patients diagnosed at 37 and 42 years). The amino-acids affected by the three mutations are highly conserved among species (Supplementary Fig.2).

**ATP sensitivity of mutant K<sub>ATP</sub> channels.** To test whether the mutations associated with T2D might affect stimulus-secretion coupling in β-cells, we next measured the sensitivity to ATP of recombinant K<sub>ATP</sub> channels carrying Y356C, R248Q and K1521N SUR1, and compared these to the ATP sensitivity of TND-associated mutants (4), L582V, H1023Y and R1379C. cDNAs encoding the above SUR1 mutants were generated by site-directed mutagenesis and co-expressed with wild-type Kir6.2 in HEK293 cells (see Research Design and Methods). Analysis of the ATP-sensitivity of the resulting K<sub>ATP</sub> channel complexes in inside-out excised patches revealed a clear correlation with the two different forms of diabetes (Fig.1C). Thus, all three TND-associated mutations tested caused a substantial (>40-fold) decrease of ATP-sensitivity. By contrast, the T2D-associated mutations had no or a much smaller effect on ATP-sensitivity. The concentration-
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inhibition curves for K\textsubscript{ATP} channels carrying SUR1-R248Q and SUR1-K1521N were practically identical to the wild-type, suggesting either that these mutations affected other properties of the channel or were not responsible for diabetes (Fig.1C). K\textsubscript{ATP} conductance of the inside-out patches expressing SUR1-K1521N was not different from wild-type (11.3±5.6nS and 12.5±5.9nS respectively), as measured in nucleotide-free solution. Patches with SUR1-R248Q channels exhibited much smaller conductances of 1.2±0.8nS. Neither of these two mutations can therefore be directly linked to the down-regulation of insulin secretion.

By contrast, K\textsubscript{ATP} channels carrying Y356C-SUR1 showed a ~four-fold decrease in ATP-sensitivity (Fig.1C). This prompted us to investigate in detail how the Y356C mutation affected the ATP-sensitivity and/or surface expression K\textsubscript{ATP} channels. We also assessed the impact of this and other mutants on stimulus-secretion coupling. Given the limited magnitude of the T2D-associated mutant’s effects we used a TND-associated SUR1 mutation, L582V (4), as a positive control.

Effect of heterozygocity and Mg\textsuperscript{2+}-dependence of the shift in ATP-sensitivity. Since all of the patients carried only a single copy of the mutated allele, we deemed it important to mimic expression of the channel in this “heterozygotic” form in single cells. To achieve this, we co-expressed the recombinant wild-type and mutant channel subunits (19,20). cDNAs encoding wild-type or mutant SUR1 were cloned into plasmids (pIRES) from which reporter proteins that have non-overlapping fluorescent emission, namely EGFP (\(\lambda_{\text{em}}=507\text{nm}\)) or dsRed2 (\(\lambda_{\text{em}}=582\text{nm}\)) were expressed from an internal ribosome entry site on the same message as SUR1. Although EGFP/dsRed emission intensity ratio may have been used, in principle, to quantify the relative expression of mutant and wild-type K\textsubscript{ATP} channels in any given cell, we assumed every EGFP/dsRed cell to be “heterozygous” (see Supplementary information for details).

The ATP sensitivity of “heterozygous” Y356C-SUR1 (“hetY356C”, Fig.2A,B, Table 2) was higher than that of “homozygous” Y356C-SUR1 (“homY356C”) channels. Thus, hetY356C and homY356C channels were each inhibited by ATP with IC\textsubscript{50}=61\,\mu\text{mol/l} and IC\textsubscript{50}=95\,\mu\text{mol/l} respectively, compared to IC\textsubscript{50}=24\,\mu\text{mol/l} for the wild-type channel. By contrast, “heterozygous” channels expressing L582V-SUR1 (“hetL582V”) were more ATP-sensitive than “homozygous” L582V-SUR1 channels (“homL582V”): IC\textsubscript{50}=869\,\mu\text{mol/l} and IC\textsubscript{50}=1140\,\mu\text{mol/l} for “het582V” and “hom582V”, respectively (Fig.2A,D, Table 2).

ATP has a dual effect on the activity of K\textsubscript{ATP} channels: it inhibits when binding to Kir6.2 but activates, in a Mg\textsuperscript{2+}-dependent manner, when binding to NBDs of SUR1 (21-25). In the wild-type channels, the former effect dominates over the physiological range of free ATP concentrations (26). Gain-of-function mutations in either subunit frequently act by enhancing the Mg\textsuperscript{2+}-dependent activation (4,27) so we tested if this was the case for Y356C-SUR1. When Mg\textsuperscript{2+} was removed from the intracellular (bath) solution, thereby abolishing Mg\textsuperscript{2+}-dependent activation, ATP blocked the wild-type channels with IC\textsubscript{50}=8\,\mu\text{mol/l}. The sensitivity of hetY356C and homY356C channels increased to IC\textsubscript{50}=25\,\mu\text{mol/l} and IC\textsubscript{50}=38\,\mu\text{mol/l} respectively (Fig.2A,C, Table 1). HetL582V and homL582V were both blocked with IC\textsubscript{50}=17\,\mu\text{mol/l}, which represents almost a 100-fold shift as compared to the Mg\textsuperscript{2+}-containing solution (Fig.2A,E, Table 1). Thus, the gain-of-function effect of L582V mutation was mediated via Mg\textsuperscript{2+}-dependent activation, while the effect of Y356C apparently occurred through a different mechanism. Y356C does not alter surface expression of K\textsubscript{ATP} channels. Transfection of HEK293
cells with cDNA encoding wild-type Kir6.2 and SUR1 subunits resulted in significant accumulation of SUR1 in the cytoplasm or cytoplasmic structures, as detected using anti-c-myc antibodies in permeabilised cells (Fig.3A). Examined in intact cells, SUR1 could also be detected on the plasma membrane (Fig.3B), consistent with previous findings in β-cells (28,29).

Introduction of the Y356C mutation into SUR1 did not affect cytoplasmic (Fig.3C) or membrane (Fig.3D) localisation. Similarly, the cytoplasmic disposition of L582V-SUR1 was not different from that of the wild-type SUR1 (Fig.3E). Interestingly, we did note a tendency towards lower cell surface expression of L582V-SUR1 (Fig.3F) vs wild-type.

Effect of glucose on electrical activity of β-cells expressing the mutant channels

In the absence of a metabolic stimulus, the membrane potential of pancreatic β-cells is largely set by the K+ conductance of KATP channels (GKATP) (30). Metabolic inhibition of GKATP depolarises the membrane logarithmically, in agreement with Goldman-Hodgkin-Katz formalism (9). Alterations in the metabolic inhibition of KATP channels may therefore affect glucose-induced electrical activity of β-cells as well as Ca2+ influx via L-type CaV channels. We therefore studied the effect on these two phenomena of the Y356C and L582V mutations in SUR1.

To this end, we over-expressed wild-type or mutant SUR1 subunits in INS1(832/13) β-cells. Over-expression of SUR1 had no effect on the level of cell surface expression of KATP channels (data not shown). We incubated cells in glucose-free extracellular solution for 30 min. and measured changes in membrane potential (Vm) and KATP conductance (GKATP), in response to increasing levels of glucose. The resting Vm of cells expressing the mutant channels (“het-” or homY356C” and ”het- or homL582V” cells respectively) was not different from that of cells expressing only wild-type SUR1 (“wt” cells): Vm=−67.4mV (wt), Vm=−67.1mV (homY356C), Vm=−67.1mV (hetY356C), Vm=−70.5mV (homL582V), Vm=−68.4mV (hetL582V). Although addition of 1mmol/l glucose had no effect on Vm in any of the three groups, further addition of 5 and then 10mmol/l glucose depolarised the membrane of wt cells and hetY356C cells (Fig.4A,B,C). By contrast, homY356C and both hom- and hetL582V showed a markedly inhibited response to increasing glucose concentrations. On the other hand, 0.2mmol/l tolbutamide depolarised all the cells (see Research Design and Methods). Subsequent perifusion with the mitochondrial uncoupler carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazon (FCCP, 2µmol/l) repolarised the plasma membrane (Fig.4B,C).

Despite the presence of an clear tendency (Fig.4B) we did not observe statistically significant differences in glucose-induced changes of the baseline membrane potential between wt and hetY356C cells. However, overall, glucose affected the electrical activity differently in the two groups. In wt cells, addition of 5, 10 and 20mM glucose depolarised the β-cell membrane and increased the frequency of action potentials (0.62±0.12Hz in 10mM glucose). Addition of 0.2mM tolbutamide produced further depolarisation, which lead to a reduction of the firing frequency (0.37±0.07Hz) and, in some cases, degeneration of the oscillations (Fig.4A,D). This effect is not typical for native β-cells, where the firing frequency increases monotonously, with the KATP conductance decreasing (31). The non-monotonous dependence has been described experimentally in α-cells (32) (and has a straightforward theoretical explanation in terms of the Morris-Lecar model of interacting ion conductances (33)). In hetY356C cells, the glucose-induced depolarisation evoked action potentials with lower frequency (0.35±0.07Hz) which was further increased by application of tolbutamide (0.41±0.10Hz). It should be stressed that 10mM glucose did not produce a larger depolarisation in hetY356C cells.
Thus, despite a relatively small shift in the ATP sensitivity of K<sub>ATP</sub> channels, β-cell lines expressing Y356C-SUR1 demonstrated impaired coupling between nutrient stimulation and electrical activity. 

Glucose fails to induce normal increases in intracellular free Ca<sup>2+</sup> in β-cell lines expressing mutant K<sub>ATP</sub> channels. To further explore the effects of the identified mutants on metabolic signal transduction we deployed single-cell Ca<sup>2+</sup> imaging of INS1(832/13) cells (Fig.5A-C; see Research Design and Methods). Examined at 3mmol/l glucose (basal) [Ca<sup>2+</sup>]<sub>cyt</sub> was stable in both wt and homY356C cells (Fig.5A). The addition of 20mmol/l glucose had different effects on the two groups: in the majority of wt cells we observed oscillations of [Ca<sup>2+</sup>]<sub>cyt</sub> which were not detected in Y356C cells. INS1(832/13) cells over-expressing K1521N-SUR1 channels showed an unchanged [Ca<sup>2+</sup>]<sub>cyt</sub> response to glucose compared to wt cells (Fig.5B,C). By contrast, glucose-stimulated [Ca<sup>2+</sup>]<sub>cyt</sub> increases were essentially abolished in cells over-expressing any of the three TND mutants (Fig.5B,C).

DISCUSSION

An important goal of the present studies was to determine whether mutations in ABCC8, identified in patients with T2D or mild disturbances in insulin secretion in adulthood, lead to changes in the molecular properties of K<sub>ATP</sub> channels and impaired stimulus-secretion coupling. In the case of Y356C SUR1 this was indeed observed. Thus, we show that a relatively small change in channel ATP sensitivity: (a) affects glucose signalling in vitro and (b) is associated with adult-onset type 2 diabetes. Importantly, we observed a clear correlation between the magnitude of the shift in ATP sensitivity for channels incorporating different SUR1 mutants, and the severity of impaired glucose metabolism in carriers, as previously demonstrated for KCNJ11/Kir6.2 mutations (6). Whilst the present study was under review, Patch et al (34) reported several mutations in ABCC8 which appear to be associated with diabetes onset in adults median age 27.5, consistent with the present findings. However, the Y356C mutation which we describe appears to cause diabetes outside the range described by (34) (maximum age recorded 35 years) and may thus represent a milder effect on channel opening.

Effect of the T2D-associated mutation on stimulus-secretion coupling.

The relatively small shift in ATP-sensitivity (from 24 to 95µmol/l as measured in inside-out patches) caused by the Y356C mutation in ABCC8/SUR1, clearly affected glucose-induced changes in β-cell electrical activity. This result strongly suggests that the Y356C mutation may lead to a diabetic phenotype. Indeed, the oral glucose tolerance test and euglycemic hyperinsulinic clamp performed in the two non diabetic carriers of the Y356C mutation showed a mild decrease of insulinogenic and disposition indexes (Table 1). This mild alteration of insulin secretion could thus lead to overt diabetes as diagnosed in their father. Similar results were reported previously for mutations in KCNJ11/Kir6.2 which affected glucose homeostasis (metabolic sensitivity) (9,10,13,14).

We observed that two mutations (Y356C and L582V) that are associated with phenotypes of different severity in heterozygous patients cause different shifts in the ATP-sensitivity of the K<sub>ATP</sub> channel (Fig. 2B vs 2D). Whilst clear differences were observed between the glucose-induced changes in V<sub>m</sub> when either channel was expressed in INS1(832/12) “heterozygously”, the “homozygous” expression of either mutant led to a near-complete suppression of depolarisation in response to glucose (Fig. 4A,B,C). By contrast, glucose-induced [Ca<sup>2+</sup>]<sub>cyt</sub> changes (Fig.5) were still clearly different for the two types of channel, even after “homozygous” expression. Thus, the Y356C mutant lead to a substantially less marked inhibition of glucose-induced [Ca<sup>2+</sup>]<sub>cyt</sub> increase than L582V (Fig. 5B). This suggests that subtle differences in V<sub>m</sub> may
be translated into more pronounced differences in $[\text{Ca}^{2+}]_{\text{cyt}}$ and, possibly, exocytosis. Alternatively, this apparent discrepancy may reflect the fact that there may be differences in the generation of $K_{\text{ATP}}$ regulators including ATP, but also the substrates/products of adenylate (23,35,36) and creatine (26,37,38) kinases, fatty acids (39) and inositol-phosphates (40,41) during electrophysiological recordings in whole-cell perforated patch (at 23°C) and in $\text{Ca}^{2+}$ imaging or secretion experiments (35-37°C).

**Molecular mechanism of Y356C effect on ATP-sensitivity.** Although the function of $K_{\text{ATP}}$ channels is well described, there is little direct experimental information on their 3-dimensional structures. Predictions based on a previously-described homology model of the Kir6.2 subunit are consistent with experimental data on its interaction with ligands (42,43), and this model has proved to be useful for interpreting the effects of mutations in $KCNJ11$ associated with neonatal diabetes (44). However, the structure of SUR1 is less well understood, because its overall level of sequence identity with the most closely-related prokaryotic homologue of known high-resolution structure, the *Staphylococcus aureus* transporter Sav1866 (45), is only ~21%. In addition, the resolution of a structural model of the channel complex, obtained by cryoelectron microscopy of a Kir6.2-SUR1 fusion protein, is only 18Å (46).

The interaction between the Kir6.2 and SUR1 subunits in this complex is predicted to be mediated by the C-terminus of Kir and TMD0 of SUR1 (47). Mutations in TMD0 (48) or the TMD0-TMD1 linker (49) can affect the $K_{\text{ATP}}$ channel gating (50) or amplify the stimulatory effect of Mg-nucleotides on SUR1 (49), thereby causing a severe diabetic phenotype in neonatal patients. However, the low resolution structure of the $K_{\text{ATP}}$ channel complex suggests that TMD1 and/or TMD2 are also likely to interact with the Kir6.2 subunit (46).

Given that tyrosine 356 is known, from the experimentally-determined topology of SUR1 (51), to be located in TMD1, it was of interest to predict its potential structural and functional roles within this domain. To this end, a homology model of a portion of SUR1 (lacking TMD0) was created, using the bacterial multidrug transporter Sav1866 (45) as a template (see Supplementary Information). In the model, Y356 is located at the extracellular end of the second transmembrane helix in TMD1, with the side-chain oriented towards the outside of the helix bundle (Supplementary Fig.3). It would thus, at least in principle, be in a position to interact with TMDs of Kir6.2 (or TMD0 of the same SUR subunit). Such an interaction, leading to an effect of the mutation on ATP sensitivity via an allosteric effect on Kir6.2 rather than involving the SUR1 NBDs, would be consistent with the observation that the removal of Mg$_{\text{2+}}$ (which abolishes the activatory effect of adenine-nucleotides on NBDs) (24) did not abolish the activatory effect of Y356C (Fig.3C).

The functional importance of Y356 is also suggested by the fact that an aromatic residue is conserved at the corresponding location not only in human SUR1 but also in MRP1, 2, 3, 4 and 6. Moreover, the TMD1 helix shows greater evolutionary conservation than the corresponding helix in TMD2, consistent with a role in protein-protein interaction rather than interaction with the lipid bilayer (data not shown).

In contrast to Y356C, the activatory effect of mutations L582V (Fig.3D) and H1023Y (4) was not observed under Mg$_{\text{2+}}$-free conditions, suggesting that these mutations exert their effects via the SUR1 NBDs. In the model, these residues, in the sixth transmembrane helix of TMD1 and the first transmembrane helix of TMD2 respectively, are more deeply buried within the protein structure and are predicted to interact with residues in TMD1 helix 3 and TMD2 helix 6 respectively (Fig.1 and Supplementary Fig.3). Disruption of such interactions by mutation might exert an allosteric effect on the nucleotide binding activity of the NBDs: the TMDs are known...
to influence the ATPase activity of these domains in SUR1 (52).

In conclusion, we demonstrate that a weakly-activating mutation in $ABCC8$ is the likely underlying cause of a heritable form of type 2 diabetes and insulin secretory insufficiency. This observation provides further evidence that quantitative shifts in the ATP sensitivity of single $K_{ATP}$ channels, in this case mediated by SUR1, can lead to broadly proportional changes in whole body glucose homeostasis (44).

ACKNOWLEDGEMENTS
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REFERENCES

FIGURE LEGENDS

Figure 1
A: Schematic of K\textsubscript{ATP} channel heterooctameric assembly B: Topological sketch of SUR1. Mutations at the residues marked are associated with T2D (*) and TND (**). The residues are indicated according to (51) and homology modeling of TMD1 and TMD2 of SUR1 (see Supplementary Info). C: ATP concentration-inhibition curves for wild-type and mutant K\textsubscript{ATP} channels measured in the presence of Mg\textsuperscript{2+} in the intracellular solution. The curves were fitted to equation 1. The mean parameters of ATP inhibition are given in the Supplementary Table 2.

Figure 2
Measurements of membrane currents. A: Currents from inside-out patches excised from HEK293 cells overexpressing recombinant Kir6.2/SUR1-WT, Kir6.2/SUR1-Y356C and Kir6.2/SUR1-L582V, in Mg\textsuperscript{2+}-containing (left) and Mg\textsuperscript{2+}-free (right) solution. Addition of 100\textmu M ATP (±Mg\textsuperscript{2+}) is indicated. Stimulation protocol is given in the inset. B-E: ATP (±Mg\textsuperscript{2+}) concentration-inhibition curves for wild-type (open circles) and “heterozygous” (half-filled circles) and “homozygous” (filled circles) mutant K\textsubscript{ATP} channels. B: MgATP concentration-inhibition curves for wild-type and Kir6.2/SUR1-Y356C K\textsubscript{ATP} channels. C: ATP (Mg\textsuperscript{2+}-free) concentration-inhibition curves for wild-type and Kir6.2/SUR1-Y356C K\textsubscript{ATP} channels. D: MgATP concentration-inhibition curves for wild-type and Kir6.2/SUR1-L582V K\textsubscript{ATP} channels. E: ATP (Mg\textsuperscript{2+}-free) concentration-inhibition curves for wild-type and Kir6.2/SUR1-L582V K\textsubscript{ATP} channels.

Figure 3
Subcellular localisation of wild-type and mutant K\textsubscript{ATP} channels: HEK cells were transfected either with c-myc-tagged SUR1 (wild-type or mutant) subunit alone or together with Kir 6.2. A c-myc tag was inserted into the extracellular loop of the SUR1 subunit. A, C and E: representative confocal images of cells expressing the SUR1 (wild-type, A, Y356C, C and L582V, E) subunit alone. Cells were fixed, permeabilised and stained with anti c-myc antibody. B, D, F: representative images of cells expressing SUR1 (wild-type, B, Y356C, D and L582V, F) subunits together with Kir6.2. Cells were directly stained with anti c-myc antibody after fixation to detect surface channels. The images are representative of 3 separate experiments, with 10 cells per condition per experiment.

Figure 4
Effect of extracellular glucose on membrane potential (A-C) and [Ca\textsuperscript{2+}]\textsubscript{cyt} (D-F) measured in β-cell lines transiently expressing wild-type and mutant K\textsubscript{ATP} channels. A: Representative recordings of membrane potential of INS1(823/13) cells expressing wild-type, hetY356C and homY356C K\textsubscript{ATP} channels. The addition of 10mmol/l glucose and 0.2mmol/l tolbutamide is indicated. B, C: dependence of the membrane potential on extracellular glucose for INS1(832/13) cells expressing: B, wild-type (open circles, n=14) hetY356C (half-filled circles, n=10) and homY356C (closed circles, n=7); C, wild-type (n=14), hetL582V (n=7) or homL582V (n=7), labels as above. The membrane potential at 0.2mM tolbutamide and 2\mu M FCCP is indicated with arrows. Statistical significance of differences between the mutant and wild-type: P<0.05 (*) and P<0.01 (**). D: Frequency of action potentials recorded from INS1(832/13) cells expressing wild-type, hetY356C and homY356C K\textsubscript{ATP} channels, in 10 mM glucose (white columns) and 0.2 mM tolbutamide (black columns). Statistical significance of differences between 10 mM glucose and 0.2 mM tolbutamide (dependent samples, Wilcoxon signed-rank test) : P<0.05 (*) and P<0.01 (**).
Figure 5
Effect of SUR1 mutations on glucose-stimulated Ca\(^{2+}\) influx into INS-1(832/13) cells. A: Representative recordings of Fura-Red fluorescence ratio (excitation 440nm/480nm) in the cytoplasm of INS1(823/13) cells expressing wild-type and homoY356C K\(_{ATP}\) channels. The addition of 3 and 20mmol/l glucose and 30mmol/l KCl is indicated with horizontal bars. B: Ratio (440/480) measured in INS1(832/13) cells expressing wild-type and mutant K\(_{ATP}\) channels, exposed to 20mM glucose. We performed n=3 experiments per mutation, with 25 – 40 cells being imaged per experiment. To correct for cell-to-cell variations, the values of ratio measured in 20mM glucose were normalised to the basal values (3mM glucose) for every cell. Statistical significance of differences between the mutant and wild-type: P<0.05 (*) and P<0.01 (**). C: Percentage of INS1(832/13) cells, expressing wild-type and mutant K\(_{ATP}\) channels, in which 20mM glucose produced an increase in the ratio (430/480), over 5% of the baseline value.
Type 2 diabetes and $K_{ATP}$ channels

**Figure 1**

A

B

C

- Wild-type
- Y356C
- K1521N
- H1023Y
- R248Q
- L582V
- R1379C

$G/G_0$ vs. [MgATP], μM
Type 2 diabetes and $K_{ATP}$ channels

**figure 2**

A

- **WT**
  - 100 $\mu$M MgATP
  - 10 $\mu$M ATP
  - 100 $\mu$M ATP
  - 1 nA
  - 10 s
  - 0.5 nA
  - 20 s

- **Y356C**
  - 100 $\mu$M MgATP
  - 10 $\mu$M ATP
  - 100 $\mu$M ATP
  - 1 nA
  - 10 s
  - 0.5 nA
  - 20 s

- **L582V**
  - 1000 $\mu$M MgATP
  - 100 $\mu$M MgATP
  - 10 $\mu$M ATP
  - 100 $\mu$M ATP
  - 0.5 nA
  - 20 s

B

- Graph showing $G/G_c$ vs. [MgATP], $\mu$M

C

- Graph showing $G/G_c$ vs. [ATP], $\mu$M

D

- Graph showing $G/G_c$ vs. [MgATP], $\mu$M

E

- Graph showing $G/G_c$ vs. [ATP], $\mu$M
Type 2 diabetes and $K_{ATP}$ channels

Figure 3

**SUR 1**

**Kir6.2/SUR1**
Type 2 diabetes and $K_{ATP}$ channels

**Figure 4**

A. 
- **Wild-type**
  - 0 mM glucose
  - 10 mM glucose
  - 2 mM tolbuthiazide

B. 
- Plot showing voltage ($V_m$, mV) vs. glucose concentration (mM)
  - 0.1 mM tolbutamide
  - 2 μM FCCP

C. 
- Plot showing voltage ($V_m$, mV) vs. glucose concentration (mM)
  - 0.1 mM tolbutamide
  - 2 μM FCCP

D. 
- Histogram showing AP frequency (Hz)
  - WT
  - hetY356C
  - homY356C
  - 10 mM glucose
  - 100 μM tolbutamide

20
Type 2 diabetes and $K_{ATP}$ channels

Figure 5
Table 1
Clinical characteristics of non diabetic carriers of the SUR1-Y356C mutation compared to normoglycemic control subjects.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age at examination, years</th>
<th>BMI kg/m²</th>
<th>Fasting blood concentrations</th>
<th>Insulinogenic index§, µUI.µmol⁻¹</th>
<th>Insulin sensitivity#, mg.kg⁻¹.min⁻¹</th>
<th>Disposition index¶, µUI.mol⁻¹</th>
<th>Glycemic Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daughter*</td>
<td>35</td>
<td>19.37</td>
<td>4.9</td>
<td>1.33</td>
<td>2.58</td>
<td>12.32</td>
<td>31.78</td>
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<tr>
<td>Son*</td>
<td>33</td>
<td>22.22</td>
<td>5.0</td>
<td>4.11</td>
<td>7.95</td>
<td>6.71</td>
<td>53.34</td>
</tr>
<tr>
<td>Control Subjects (n = 18)</td>
<td>26.79 ± 6.56</td>
<td>22.9 ± 3.3</td>
<td>4.6 ± 0.3</td>
<td>5.5 ± 3.8</td>
<td>16.9±10.7</td>
<td>10.88±2.36</td>
<td>184.21±25.16</td>
</tr>
</tbody>
</table>

§ Insulinogenic index was calculated from a 75-g oral glucose tolerance test: Insulinemia T30’-Insulinemia T0/ Glycemia T30’-Glycemia T0
# Insulin sensitivity index was evaluated as the M value during an euglycemic hyperinsulinic clamp.
¶ Disposition index was calculated as a measure of β-cell function: M value x insulinogenic index (53).
* Daughter and son of the diabetic proband identified with the Y356C mutation. The proband himself has been treated with glibenclamide for several years. This sulfonylurea molecule acts to improve insulin secretion through closure of SUR1/Kir6.2 channels in pancreatic β-cells. Consequently, exploration of insulin secretion parameters in this treated patient would be uninterpretable.
Table 2
Parameters of ATP inhibition for $K_{ATP}$ channels with mutant SUR1 subunit. Numbers of experiments are given in parentheses. Difference with the respective parameter for wt, significant at $p<0.05$ (*) and $p<0.01$ (**).

<table>
<thead>
<tr>
<th>Mg^{2+} mmol/l</th>
<th>IC_{50}</th>
<th>WT “hetero”</th>
<th>Y356C “hetero”</th>
<th>Y356C “homo”</th>
<th>L582V “hetero”</th>
<th>L582V “homo”</th>
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<tr>
<td>2</td>
<td>24±3 (5)</td>
<td>61±11 (10)**</td>
<td>95±9 (10)**</td>
<td>869±48 (6)**</td>
<td>1140±346 (6)**</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>8±1 (6)</td>
<td>25±5 (7)**</td>
<td>38±8 (8)**</td>
<td>17±3 (5)*</td>
<td>17±3 (6)*</td>
<td></td>
</tr>
</tbody>
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