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A rare mutation in *ABCC8*/SUR1 leading to altered K_{ATP} 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_{ATP}) 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_{ATP} 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_{ATP} channel activity was measured in inside-out patches, and plasma membrane potential in perforated whole-cell patches. Cytoplasmic [Ca^{2+}] 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_{ATP} channels incorporating Y356C-SUR1 displayed lower sensitivity to MgATP (IC_{50}=24μmol/l and 95μmol/l for wild-type and mutant channels, respectively). Similar effects were observed in the absence of Mg^{2+}, suggesting an allosteric effect via associated Kir6.2 channels. Over-expression of Y356C-SUR1 in

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INS1(832/13) cells impaired glucose-induced cell depolarisation and increases in intracellular free Ca^{2+} concentration, albeit more weakly than neonatal diabetes-associated SUR1 mutants.

Conclusions—An *ABCC8*/SUR1 mutation with relatively minor effects on K_{ATP} channel activity and β-cell glucose sensing causes diabetes in adulthood. These data suggest a close correlation between altered SUR1 properties and clinical phenotype.

Keywords

Channel Gating; Genetic Mechanism; Beta Cell Secretion

Introduction

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_{ATP}) channels. This, in turn, leads to plasma membrane depolarisation, Ca^{2+} influx and the exocytosis of dense core secretory vesicles (1;2).

K_{ATP} channels exist as heterooctamers (3) comprising four pore-forming (Kir6.2) and four regulatory (SUR1) subunits (Fig.1A), encoded by *KCNJ11* and *ABCC8* 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_{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 previously been described, although a heterozygous E1506K substitution causes hyperinsulinism in infancy, loss of insulin secretory capacity in early adulthood, and diabetes in middle-age (15)

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_{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_{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), and 17

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young probands diagnosed with MODY from the French families without known MODY-associated mutations 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 pIRES2, 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. We used pIRES2-EGFP and/or pIRES2-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 pIRES2-SUR1 cDNA in 7:3 ratio (HEK293 cells) or pIRES2-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 (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.3A), filtered at 0.15kHz and digitised at 0.5kHz. If the level of channel expression was low, K_{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 control 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: $G/G_c = 1/(1+([ATP]/IC_{50})h)$, 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) β-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 ≥2 min. Cells that were not responsive to tolbutamide were excluded. 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_{ATP} conductance (GK_{ATP}) (9). This controlled for the leaks of the patch and to verified that the depolarisation (hyperpolarisation) of the membrane was linked to K_{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 ±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 $MgCl_2$, 2.6 $CaCl_2$. The intracellular (bath) solution contained (mmol/l): 107 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 11 EGTA, 10 HEPES (pH 7.2 with KOH), plus MgATP as indicated. Mg^{2+}-free intracellular solutions contained (mmol/l): 107 KCl, 1 $CaCl_2$, 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 K_2SO_4, 10 NaCl, 10 KCl, 1 $MgCl_2$, 5 HEPES (pH7.35 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 $CaCl_2$, 1.1 $MgCl_2$. All experiments were conducted at 21-23ºC and the bath solution was perfused continuously.

Measurements of cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$)

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 NaH_2PO_4, 0.5 $MgSO_4$, 2.0 $NaHCO_3$, 3 glucose, 10 Hepes (pH7.4 with NaOH) and 1.5 $CaCl_2$ equilibrated with O_2/CO_2 (95:5, v/v) at 37°C. Changes in $[Ca^{2+}]_{cyt}$ were monitored at 0.2Hz, using a Cell^R™ (Olympus) epifluorescence imaging system, based around an Olympus Axiovert IX-81 inverted optics microscope fitted with a x40 oil immersion objective. Cells were continuously perifused in KRB solution at the glucose concentrations indicated. The “KCl” solution comprised KRB in which 50mmol/l NaCl was substituted by KCl. $[Ca^{2+}]_{cyt}$ was expressed as the ratio of fluorescence intensity (λ_{em}=597nm, λ_{ex}=440nm) to that at λex=490nm, after subtraction of background fluorescence. All experiments were performed at 37°C. To account for the differences in $[Ca^{2+}]_{cyt}$ between individual cells, the fluorescence ratios in each trace were normalised to the average ratio observed at 3mmol/l glucose. Cells were selected by expression of the reporter proteins.

Human Growth Hormone secretion assay

INS1(832/13) cells were seeded at a density of (4–6)×10^5/ml and cultured for 16 - 24h. Cells were then co-transfected with 0.5μg of hGH-encoding plasmid pXGH5 (19) together with 1μg of wild-type or mutant SUR1. hGH release was assayed 2-3 days after transfection. Cells were incubated for 30 min. in 0.5ml of KRB solution (see above). The supernatant was removed and replaced with low-glucose (3mM) KRB. Cells were then treated for 30 min. under the conditions indicated. The supernatant was removed and replaced with high-glucose (20 mmol/l) and monomethylsuccinate (5 mmol/l) KRB, for a further 45 min as shown. Supernatant was then removed and cells lysed with 500 μl of 1.0% (v/v) Triton X-100. hGH quantification was done by colorimetric sandwich ELISA assay (Roche Diagnostics Ltd).

Immunocytochemistry

Cells were transfected with pIRES2-SUR1-c-*myc* or pIRES2-SUR1-c-*myc* plus pcDNA3Kir6.2. 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 2 h 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. Statistical significance was estimated using non-parametric 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 including 17 probands with MODY from French families without known MODY-associated mutations. One of the patients with normal BMI, diagnosed with hyperglycemia at age 39 and who 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_{ATP} channel (Fig.1B). 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). 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) and K1521N (two T2D patients diagnosed at 37 and 42 years).

ATP sensitivity of mutant K_{ATP} 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_{ATP} 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_{ATP} 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-inhibition curves for K_{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_{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_{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_{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^{2+}-dependence of the shift in ATP-sensitivity

A more clinically-relevant estimate of ATP sensitivity is made by imitating the heterozygous state *in vitro*. To achieve this, we co-expressed the recombinant wild-type and mutant channel subunits (20; 21). To visualise dual ('heterozygous') expression we cloned wild-type and mutant SUR1 nucleotide sequences into pIRES plasmids with reporter proteins that have non-overlapping fluorescent emission, EGFP (λ_{em}=507nm) and dsRed2 (λ_{em}=582nm). Although EGFP/dsRed emission intensity ratio may be used, in principle, to quantify the relative expression of mutant and wild-type K_{ATP} channels in an 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_{50}=61μmol/l and IC_{50}=95μmol/l respectively, compared to IC_{50}=24μmol/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_{50}=869μmol/l and IC_{50}=1140μmol/l for 'het582V' and 'hom582V', respectively (Fig.2A,D, Table 2)

ATP has a dual effect on the activity of K_{ATP} channels: it inhibits when binding to Kir6.2 but activates, in a Mg^{2+}-dependent manner, when binding to NBDs of SUR1 (22–26). In the wild-type channels, the former effect dominates over the physiological range of free ATP concentrations (27). Gain-of-function mutations in either subunit frequently act by enhancing the Mg^{2+}-dependent activation (4;28) so we tested if this was the case for Y356C-SUR1. When Mg^{2+} was removed from the intracellular (bath) solution, thereby abolishing Mg^{2+}-dependent activation, ATP blocked the wild-type channels with IC_{50}=8μmol/l. The sensitivity of hetY356C and homY356C channels increased to IC_{50}=25μmol/l and IC_{50}=38μmol/l respectively (Fig.2A,C, Table 1). HetL582V and homL582V were both blocked with IC_{50}=17μmol/l, which represents almost a 100-fold shift as compared to the Mg^{2+}-containing solution (Fig.2A,E, Table 1). Thus, the gain-of-function effect of L582V mutation was mediated via Mg^{2+}-dependent activation, while the effect of Y356C apparently occurred through a different mechanism.

Y356C does not alter surface expression of K_{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 (29;30).

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 K_{ATP} channels (G_{KATP}) (31). Metabolic inhibition of G_{KATP} depolarises the membrane logarithmically, in agreement with Goldman-Hodgkin-Katz formalism (9). Alterations in the metabolic inhibition of K_{ATP} channels may therefore affect glucose-induced electrical activity of β-cells as well as Ca^{2+} influx via L-type Ca_V 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 K_{ATP} channels (data not shown). We incubated cells in glucose-free extracellular solution for 30 min. and measured changes in membrane potential (V_m) and K_{ATP} conductance (G_{KATP}), in response to increasing levels of glucose. The resting V_m 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): V_m=-67.4mV (wt), V_m=-67.1mV (homY356C), V_m=-67.1mV (hetY356C), V_m=-70.5mV (homL582V), V_m=-68.4mV (hetL582V). Although addition of 1mmol/l glucose had no effect on V_m in any of the three groups, further addition of 5 and then 10 mmol/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).

Resting G_{KATP} was much smaller than that reported for native β- (32) and INS1 cells (9) and highly variable from cell to cell (G_{KATP}=0.46±0.38nS/pF). However, the overall tendency of changes in this parameter was similar to that for V_m: the remaining G_{KATP} in 5, 10 and 20mmol/l glucose was smaller in wild-type and hetY356C cells, as compared to homY356C and homL582V (Supplementary Fig 4). Thus, despite a relatively small shift in the ATP sensitivity of K_{ATP} channels, β-cell lines expressing Y356C-SUR1 demonstrated impaired coupling between a nutrient stimulation and electrical activity.

Glucose fails to induce normal increases in intracellular free Ca^{2+} in β-cell lines expressing mutant K_{ATP} channels

To further explore the effects of the identified mutants on metabolic signal transduction we deployed single-cell Ca^{2+} imaging of INS1(832/13) cells (Fig.4D-F; see Research Deisgn and Methods). Examined at 3mmol/l glucose (basal) $[Ca^{2+}]_{cyt}$ was stable in both wt and homY356C cells (Fig.4D,E). The addition of 20 mmol/l glucose had different effects on the two groups: in the majority of wt cells we observed oscillations of $[Ca^{2+}]_{cyt}$ which were not detected in Y356C cells. HomY356C cells, however, exhibited a tendency towards an elevated basal $[Ca^{2+}]_{cyt}$. INS1(832/13) cells over-expressing K1521N-SUR1 channels showed an even larger $[Ca^{2+}]_{cyt}$ response to glucose than wt cells (Fig.4F). By contrast, glucose-stimulated $[Ca^{2+}]_{cyt}$ increases were essentially abolished in cells over-expressing any of the three TND mutants (Fig. 4F).

Effect of glucose on hGH secretion from INS1(832/13) cells

When INS1(832/13) cells were co-transfected with human growth hormone (hGH) along with wild-type or mutant SUR1 subunits they failed to demonstrate any glucose-stimulated release of co-transfected hGH (data not shown) (33), presumably reflecting the partial dedifferentiation of these cells (9). However, we did observe the stimulated release of this insulin surrogate in response to 20 mmol/l glucose plus 5 mmol/l methyl succinate. The latter response was unaffected in homY356C cells (Fig.5). In contrast, hGH secretion in response to the above mixed stimulus was suppressed in L582V- or H1023Y-SUR1-expressing cells (Fig.5), consistent with the more potent effects of the latter mutations on changes in membrane potential and $[Ca^{2+}]_{cyt}$ (see above; Fig. 4).

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_{ATP} 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).

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_{ATP} channel (Fig. 2B vs 2D). Whilst clear differences were observed between the glucose-induced changes in V_m 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. 4B,C). By contrast, glucose-induced $[Ca^{2+}]_{cyt}$ changes (Fig 4D-F) were still clearly different for the two channels, even after 'homozygous' expression. Thus, the Y356C mutant lead to a substantially less marked inhibition of glucose-induced $[Ca^{2+}]_{cyt}$ increase than L582V (Fig. 4F), and only the latter affected glucose-induced hGH release from INS1(832/13) cells (Fig. 5). This suggests that subtle differences in V_m may be translated into more pronounced differences in $[Ca^{2+}]_{cyt}$ and exocytosis. Alternatively, this apparent discrepancy may reflect the fact that there may be differences in the generation of K_{ATP} regulators including ATP, but also the substrates/products of adenylate (24;34;35) and creatine (27;36;37) kinases, fatty acids (38) and inositol-phosphates (39;40) during electrophysiological recordings in whole-cell perforated patch (at 23ºC) and in Ca^{2+} imaging or secretion experiments (35-37ºC).

Molecular mechanism of Y356C effect on ATP-sensitivity

Although the function of K_{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 (41;42), and this model has proved to be useful for interpreting the effects of mutations in *KCNJ11* associated with neonatal diabetes (43). 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 (44), 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 (45).

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 (46). Mutations in TMD0 (47) or the TMD0-TMD1 linker (48) can affect the K_{ATP} channel gating (49) or amplify the stimulatory effect of Mg-nucleotides on SUR1 (48), thereby causing a severe diabetic phenotype in neonatal patients. However, the low resolution structure of the K_{ATP} channel complex suggests that TMD1 and/or TMD2 are also likely to interact with the Kir6.2 subunit (45).

Given that tyrosine 356 is known, from the experimentally-determined topology of SUR1 (50), 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 (44) 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 (Figure 1, Supplementary Information). 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^{2+} (which abolishes the activatory effect of adenine-nucleotides on NBDs) (25) 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^{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 (Figure 1 and Supplementary information). 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 (51).

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 (43).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

$[Ca^{2+}]_{cyt}$	cytosolic free Ca^{2+} concentration
KRB	Modified Krebs' Ringer bicarbonate medium
PND, TND	permanent and transient neonatal diabetes mellitus, respectively
T2D	type 2 diabetes
wt	wild-type

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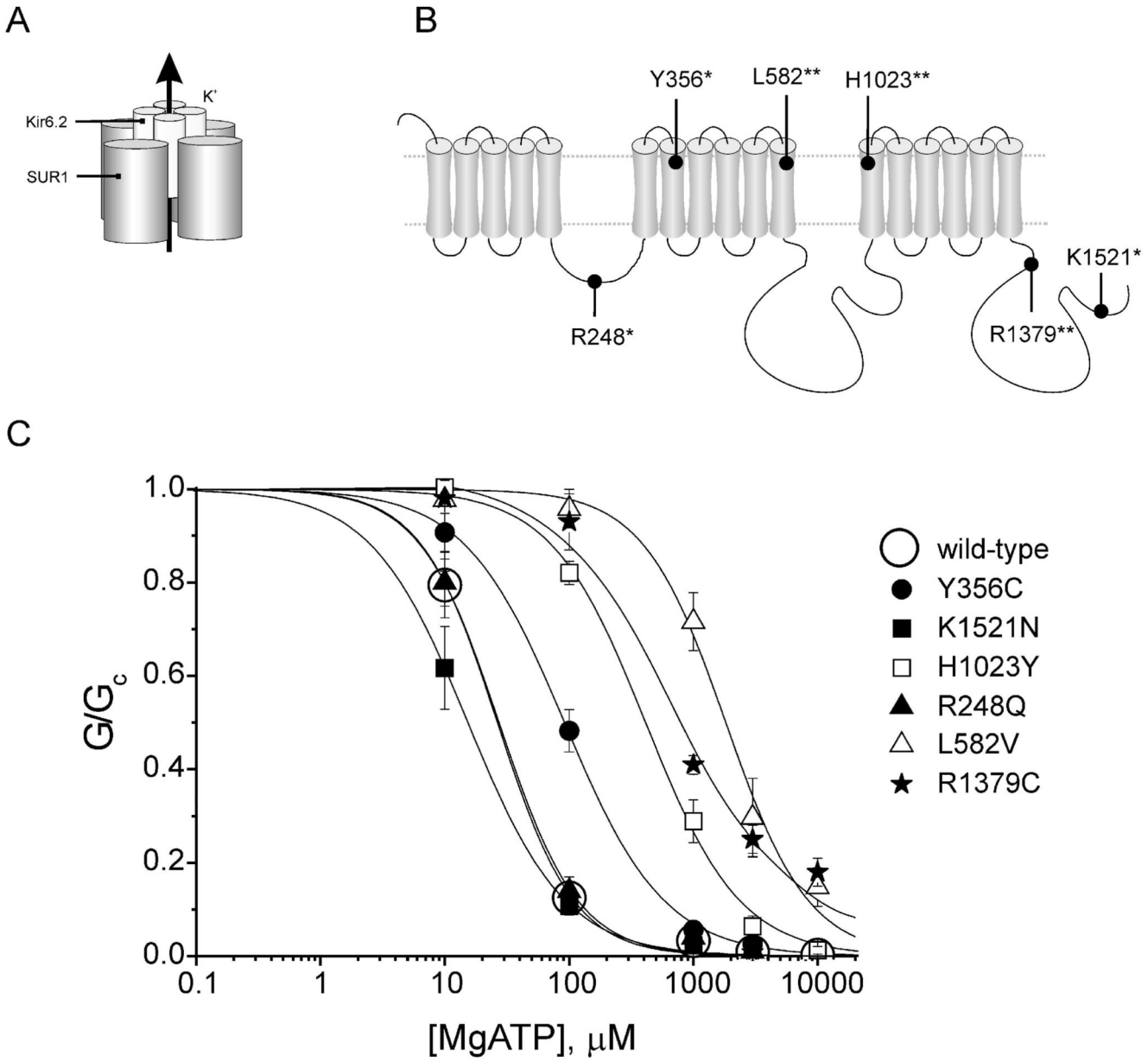

Figure 1. A: Schematic of K_{ATP} channel heterooctameric assembly B: Topological sketch of SUR1.

Mutations at the residues marked are associated with T2D (*), TND (**) and MODY (***). The residues are indicated according to (50) and homology modeling of TMD1 and TMD2 of SUR1 (see Supplementary Info). **C:** ATP concentration-inhibition curves for wild-type and mutant K_{ATP} channels measured in the presence of Mg^{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.

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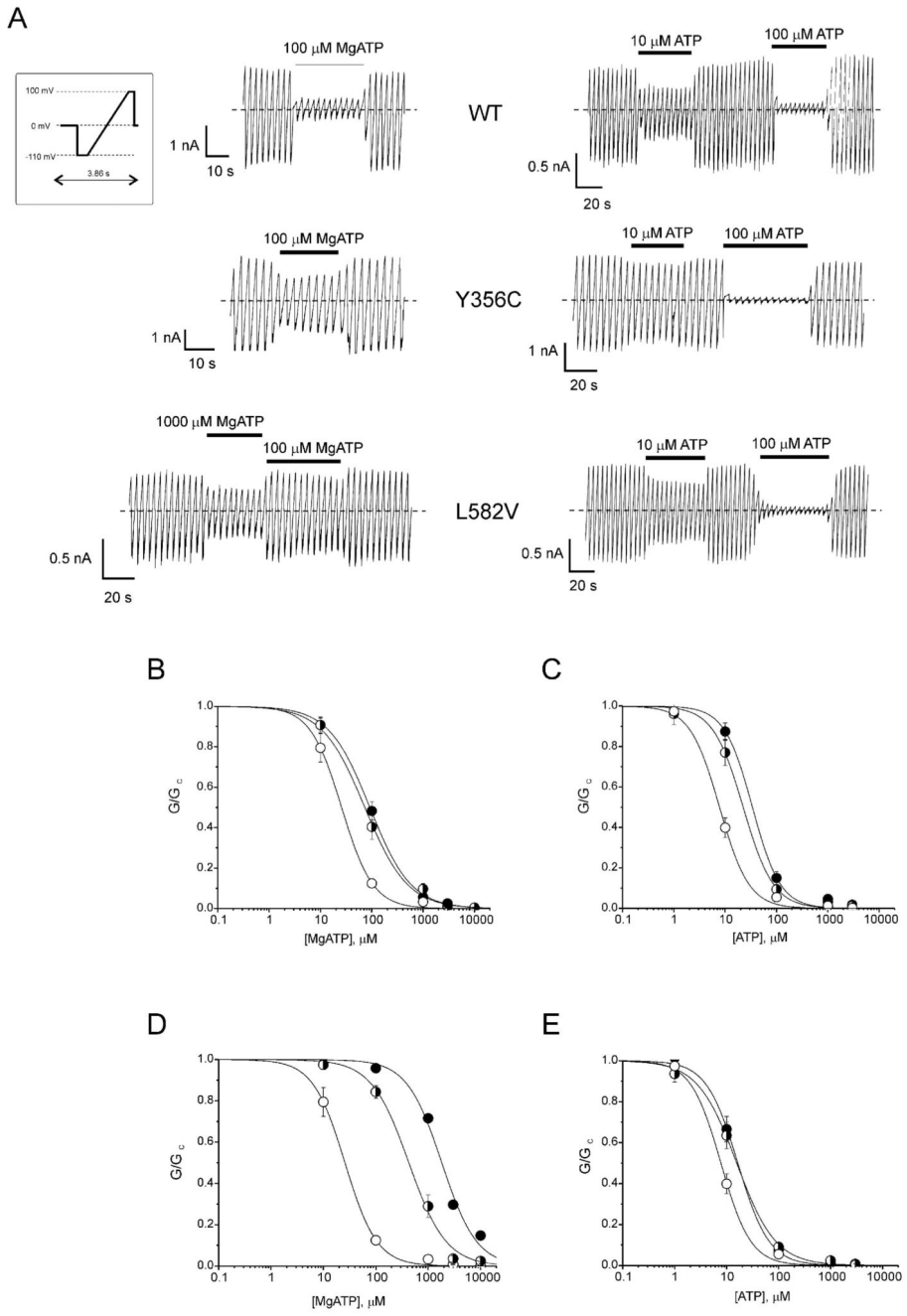

Figure 2. Measurements of membrane currents.

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^{2+}-containing (left) and Mg^{2+}-free (right) solution. Addition of 100μM ATP ($\pm Mg^{2+}$) is indicated. Stimulation protocol is given in the inset.

B-E: ATP ($\pm Mg^{2+}$) concentration-inhibition curves for wild-type (open circles) and 'heterozygous' (half-filled circles) and 'homozygous' (filled circles) mutant K_{ATP} channels.

B: MgATP concentration-inhibition curves for wild type and Kir6.2/SUR1-Y356C K_{ATP}

channels. C: ATP (Mg^{2+}-free) concentration-inhibition curves for wild type and Kir6.2/SUR1-Y356C K_{ATP} channels. D: MgATP concentration-inhibition curves for wild type and Kir6.2/SUR1-L582V K_{ATP} channels. E: ATP (Mg^{2+}-free) concentration-inhibition curves for wild type and Kir6.2/SUR1-L582V K_{ATP} channels.

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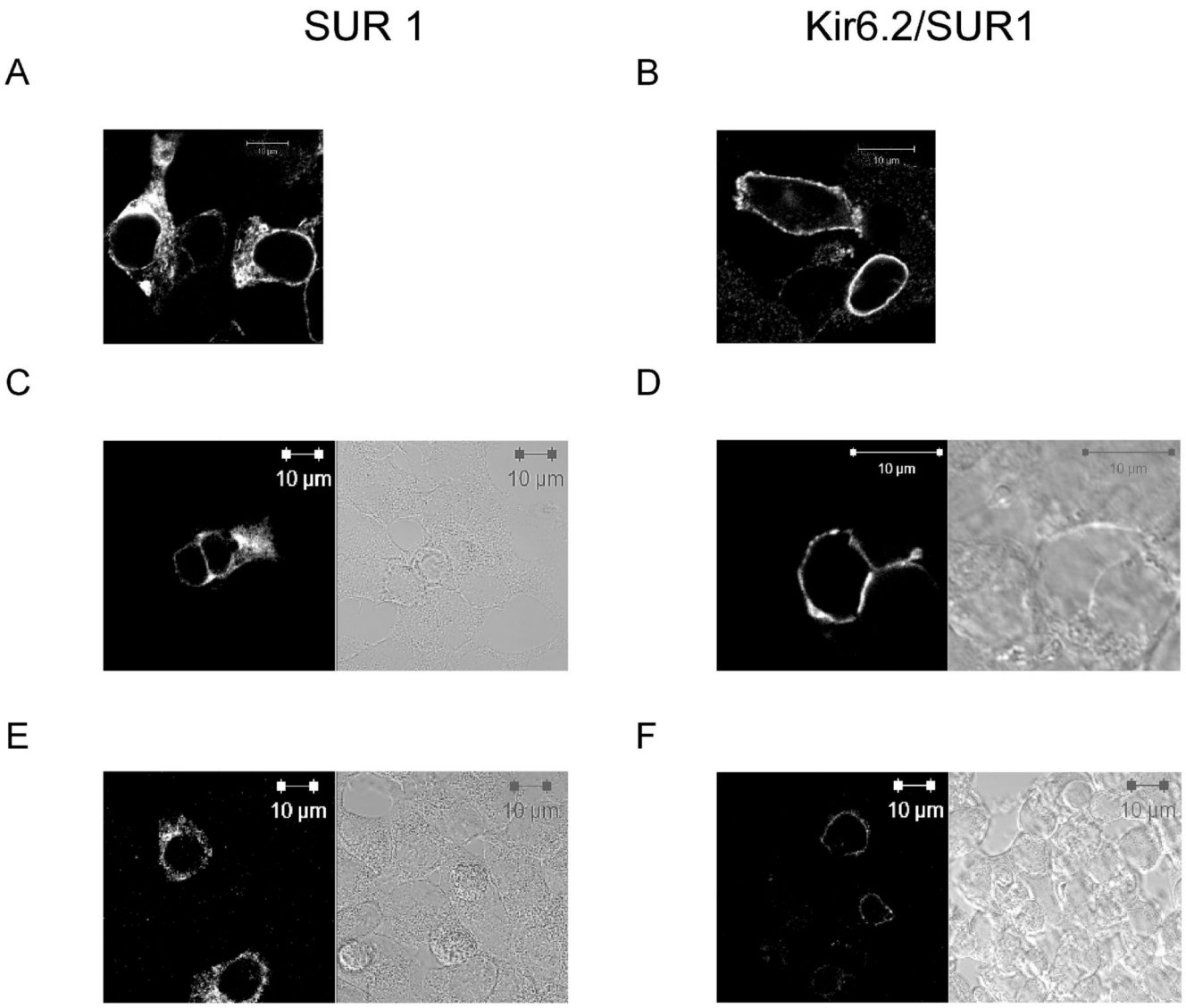

Figure 3. Subcellular localisation of wild type and mutant K_{ATP} channels:

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. Figures 2A, C and E show representative confocal images of cells expressing the SUR1 (Wt/Mut) subunit alone. Cells were fixed, permeabalised and stained with anti c-*myc* antibody. Figures 2B, D and F show representative images of cells expressing SUR1 (Wt/Mut) subunits together with Kir6.2. Cells were directly stained with anti c-*myc* antibody after fixation to detect surface channels.

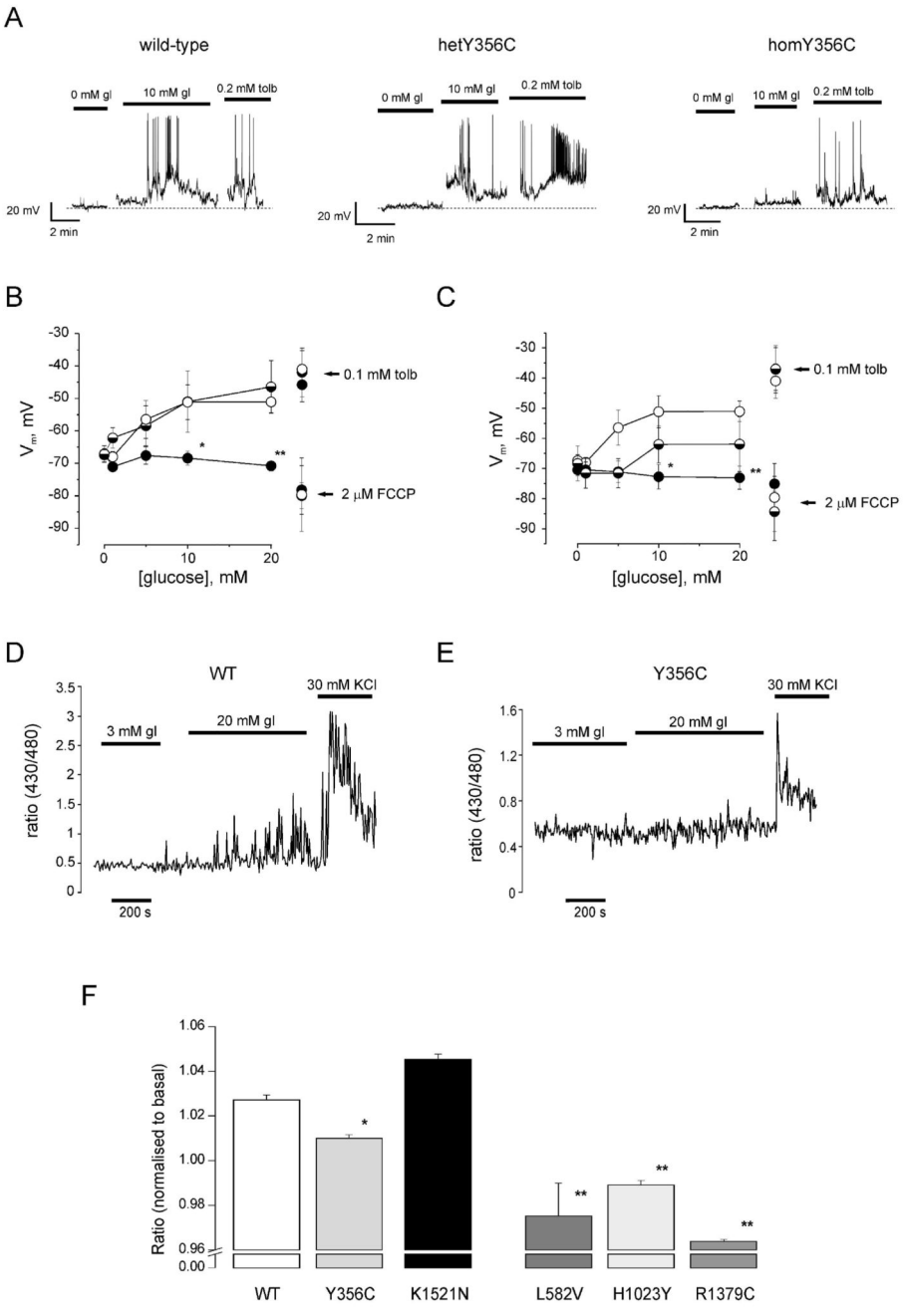

Figure 4. Effect of extracellular glucose on membrane potential (A-C) and $[Ca^{2+}]_{cyt}$ (D-F) measured in β-cell lines transiently expressing wild-type and mutant K_{ATP} channels.

A: Representative recordings of membrane potential of INS1(823/13) cells expressing wild-type, homY356C and homL582V K_{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 wild-type (open circles) hetY356C (half-filled circles) and homY356C (closed circles) (**B**) or wild-type, hetL582V or homL582V (labels as above) (**C**). The wild-type data is given as open circles, the data from

homomeric mutant is given in closed circles. The membrane potential at 0.2mM tolbutamide and 2μM FCCP is indicated with arrows.

Statistical significance of differences between the sample and wild-type: $P<0.05$ (*) and $P<0.01$ (**).

D, E: Representative recordings of Fura-Red fluorescence ratio (ex440/480)

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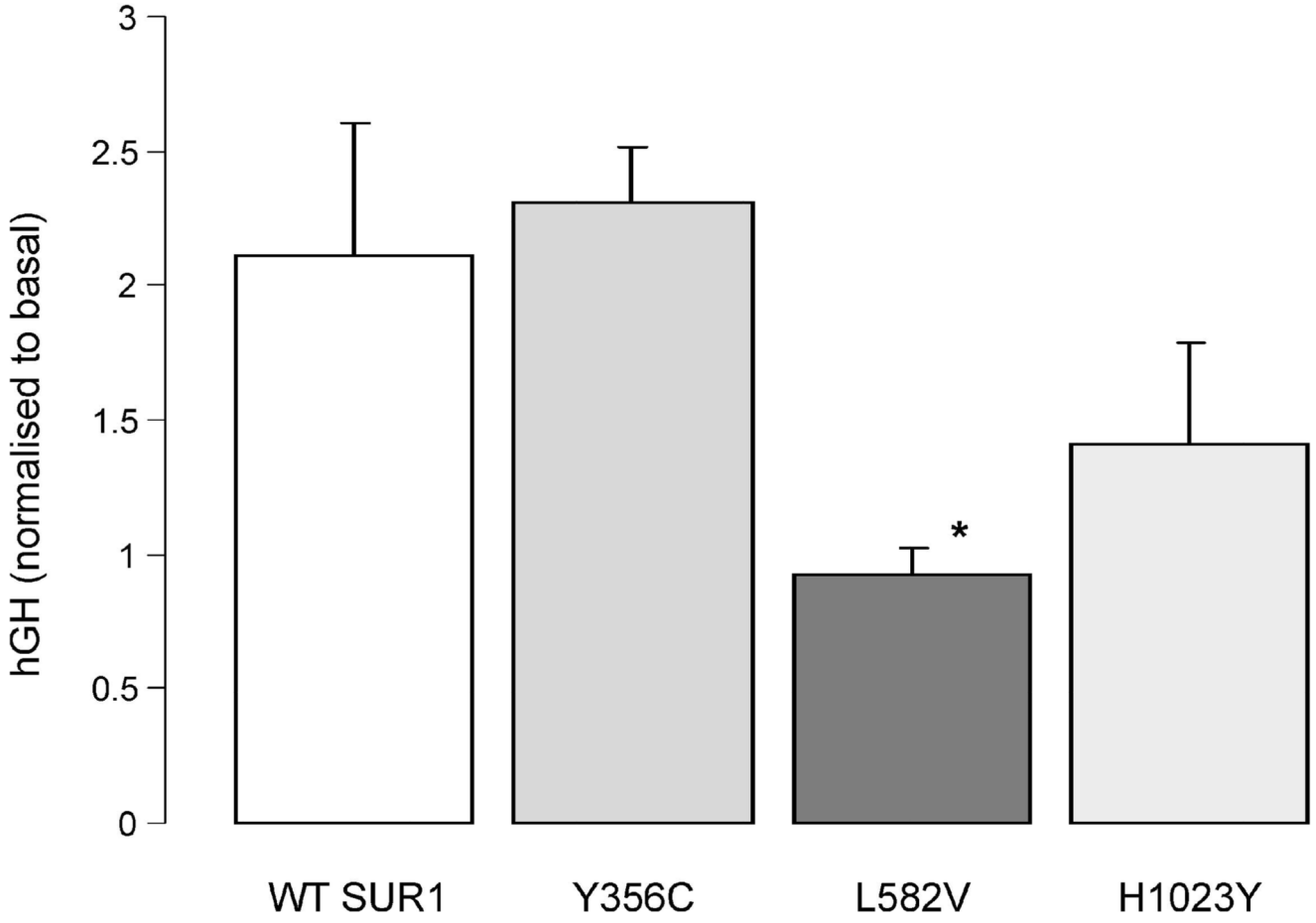

Figure 5. Effect of SUR1 mutations on glucose-stimulated hGH release from INS-1(832/13) cells.
Cells were co-transfected with 0.5 μg of hGH-encoding plasmid pXGH5 together with 1 μg of SUR1 wt or mutant. hGH release was expressed as a percentage of the total hGH and was compared with values obtained under basal conditions (3mmol/l glucose).

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Table 1

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

Subject	Age at examination, years	BMI kg/m^2	Fasting blood concentrations		Insulinogenic index[§], µUI.µmol^{-1}	Insulin sensitivity[#], mg.kg^{-1}.min^{-1}	Disposition index[¶], µUI.mol^{-1}	Glycemic Status
			Glucose, mmo/l	Insulin, µU/ml				
Daughter[*]	35	19.37	4.9	1.33	2.58	12.32	31.78	Non-diabetic
Son[*]	33	22.22	5.0	4.11	7.95	6.71	53.34	Non-diabetic
Control Subjects (n = 18)	26.79 ± 6.56	22.9 ± 3.3	4.6 ± 0.3	5.5 ± 3.8	16.9±10.7	10.88±2.36	184.21±25.16	Non-diabetic

[§]Insulinogenic index was calculated from a 75-g oral glucose tolerance test: Insulinemia T30'-Insulinemia T0/ Glycemia T30'-Glycemia T0

[#]Insulin sensivity 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 (52).

[*]Daughter and son of the diabetic proband identified with the Y356C mutation.

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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 $p<0.01$ (**).

	Mg^{2+} mmol/l	WT	Y356C		L582V	
			'hetero'	'homo'	'hetero'	'homo'
IC_{50}	2	24±3 (5)	61±11 (10)**	95±9 (10)**	869±48 (6)**	1140±346 (6)**
	0	8±1 (6)	25±5 (7)**	38±8 (8)**	17±3 (5)*	17±3 (6)*