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ARTICLE

Hyperinsulinism in mice with heterozygous loss of K_{ATP} channels

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Abstract
Aims/hypothesis ATP-sensitive K^+ (K_{ATP}) channels couple glucose metabolism to insulin secretion in pancreatic beta cells. In humans, loss-of-function mutations of beta cell K_{ATP} subunits (SUR1, encoded by the gene *ABCC8*, or Kir6.2, encoded by the gene *KCNJ11*) cause congenital hyperinsulinaemia. Mice with dominant-negative reduction of beta cell K_{ATP} (Kir6.2[AAA]) exhibit hyperinsulinism, whereas mice with zero K_{ATP} ($Kir6.2^{-/-}$) show transient hyperinsulinaemia as neonates, but are glucose-intolerant as adults. Thus, we propose that partial loss of beta cell K_{ATP} in vivo causes insulin hypersecretion, but complete absence may cause insulin secretory failure.
Materials and methods Heterozygous $Kir6.2^{+/-}$ and $SUR1^{+/-}$ animals were generated by backcrossing from knockout animals. Glucose tolerance in intact animals was determined following i.p. loading. Glucose-stimulated insulin secretion (GSIS), islet K_{ATP} conductance and glucose dependence of intracellular Ca^{2+} were assessed in isolated islets.
Results In both of the mechanistically distinct models of reduced K_{ATP} ($Kir6.2^{+/-}$ and $SUR1^{+/-}$), K_{ATP} density is reduced by ~60%. While both $Kir6.2^{-/-}$ and $SUR1^{-/-}$ mice are glucose-intolerant and have reduced glucose-stimulated insulin secretion, heterozygous $Kir6.2^{+/-}$ and $SUR1^{+/-}$ mice show enhanced glucose tolerance and increased GSIS, paralleled by a left-shift in glucose dependence of intracellular Ca^{2+} oscillations.
Conclusions/interpretation The results confirm that incomplete loss of beta cell K_{ATP} in vivo underlies a hyperinsulinaemic phenotype, whereas complete loss of K_{ATP} underlies eventual secretory failure.

Keywords *ABCC8* · Hyperinsulinism · K^+ current · K_{ATP} · *KCNJ11* · Kir6.2 · Knockout · Mice · Pancreas · SUR1

Abbreviations

$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
CHI	congenital hyperinsulinaemia
GSIS	glucose-stimulated insulin secretion
GTT	glucose tolerance test
K_{ATP} channels	ATP-sensitive K^+ channels
KO	knockout
MI	metabolic inhibitor
PNDM	permanent neonatal diabetes mellitus

Introduction

Glucose metabolism increases the cytoplasmic [ATP]:[ADP] ratio in beta cells, which causes closure of ATP-

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sensitive K^+ (K_{ATP}) channels, beta cell membrane depolarisation, opening of voltage-dependent Ca^{2+} channels and Ca^{2+} influx. The resultant rise in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) triggers insulin secretion. Thus, the K_{ATP} channel links metabolic alterations to the electrical activity of the beta cells. Naively, reduced or absent K_{ATP} channel activity is expected to result in constitutive membrane depolarisation, elevated $[Ca^{2+}]_i$ and hypersecretion of insulin. In humans, heterozygous loss-of-function mutations of beta cell K_{ATP} subunits (SUR1, encoded by the gene *ABCC8*, and Kir6.2, encoded by the gene *KCNJ11*) underlie congenital hyperinsulinaemia (CHI) [1–7], a rare genetic disease characterised by relative hyperinsulinaemia, which generally presents with high insulin levels in parallel with low blood glucose [8].

K_{ATP} channels are an obligate complex of Kir6.2 and SUR1 subunits [9–11]. Mice completely lacking Kir6.2 or SUR1 [12–14], as well as mice expressing a dominant-negative Kir6.2 transgene in beta cells (Kir6.2[AAA] [15] or Kir6.2[G132S] [16]) have been generated. Kir6.2[AAA] mice (which exhibit complete loss of K_{ATP} channels in ~70% of beta cells, but normal channel density in the remainder) show hyperinsulinaemia, enhanced glucose tolerance and increased glucose-stimulated insulin secretion (GSIS) [15]. However, mice completely lacking K_{ATP} activity ($Kir6.2^{-/-}$) show transient hyperinsulinaemia as neonates, but both $Kir6.2^{-/-}$ and $SUR1^{-/-}$ unexpectedly show glucose intolerance and loss of insulin secretion as adults [12–14, 17]. Thus, genetic abolition of beta cell K_{ATP} channels in mice fails to recapitulate some features of CHI, whereas a transgenic model of reduced K_{ATP} does reiterate a hyperinsulinaemic phenotype throughout life. We have therefore proposed that, while complete absence may cause insulin secretory failure as in Kir6.2 and SUR1 knockout (KO) mice [12, 13, 17, 18], partial loss of beta cell K_{ATP} channel activity in vivo, as observed in dominant-negative Kir6.2[AAA] mice, causes insulin hypersecretion. Here we further test this hypothesis, and show that the reduced K_{ATP} gene dosage in heterozygous $Kir6.2^{+/-}$ and $SUR1^{+/-}$ mice causes reduced K_{ATP} and insulin hypersecretion without progression to secretory failure.

Materials and methods

Generation of transgenic mice $Kir6.2^{-/-}$ mice were originally generated by targeted disruption of the gene encoding for Kir6.2 in an outbred genetic strain using E14 embryonic stem (ES) cells established from the 129Sv mouse strain (a gift from S. Seino and T. Miki, Division of Cellular and Molecular Medicine, Kobe University Graduate School of Medicine, Kobe, Japan) [14]. $SUR1^{-/-}$ mice (a gift from M. Magnuson and C. Shiota, Vanderbilt University School of Medicine, Department of Molecular Physiology and Biophysics, Nashville, TN, USA) were originally generated by microinjection of two different ES cell clones into C57/Bl6 blastocysts. The $Sur1^{lox+neo}$ allele was converted to the $Sur1^{neo}$ allele by pronuclear microinjection of *CMV-cre* expression vector (pBS185) into embryos obtained from matings of $Sur1^{lox+neo/w}$ and $Sur1^{w/w}$ mice [12]. Heterozygous mice were generated by cross-breeding of $Kir6.2^{-/-}$ (from generations >F5 backcrossed to C57/Bl6; Jackson Laboratories, Bar Harbor, ME, USA) or $SUR1^{-/-}$ and C57/Bl6 (wild-type) mice. These heterozygous $Kir6.2^{+/-}$ or $SUR1^{+/-}$ mice were then interbred to generate wild-type, heterozygous and KO offspring that were compared in the experiments. Mice were typed using primers for the wild-type Kir6.2 or SUR1 genes and primers against the neomycin-resistance gene that was incorporated in the Kir6.2 or SUR1 gene disruption [12, 14]. Kir6.2[AAA] were generated and maintained on the C57/Bl6 background [15].

Isolation of pancreatic islets and beta cells All experiments were performed in compliance with the relevant laws and institutional guidelines, and were approved by the Washington University Animal Studies Committee. Mice were anaesthetised with 2-bromo-2-chloro-1,1,1-trifluoroethane in an anaesthetising chamber and killed by cervical dislocation. Pancreata were cannulated and injected with Hank's balanced salt solution (Sigma, St Louis, MO, USA) containing collagenase (Type XI [Sigma]) (0.3 mg/ml, pH 7.4) [18]. Briefly, pancreata were digested for 5 min at 37°C, hand shaken and washed three times in cold Hank's solution [18]. Islets were manually isolated under a dissecting microscope, and maintained overnight in CMRL medium (Gibco BRL) containing 5.6 mmol/l glucose in a humidified 37°C incubator. For electrophysiological measurements, islets were washed three times in Minimal Essential Medium (without L-glutamine), followed by one wash in DMEM supplemented with trypsin/EDTA (0.01/0.002%), and a final wash in DMEM [15]. Trypsin-treated islets were dispersed into isolated cells by resuspending gently in complete CMRL medium (supplemented with FCS, 10%), penicillin (100 U/ml) and streptomycin (100 μg/ml). Isolated cells were plated on glass coverslips.

Electrophysiological measurements Macroscopic currents from dispersed islet cells were recorded using a standard whole-cell voltage-clamp configuration [15]. Electrodes were filled with K-INT (140 mmol/l KCl, 10 mmol/l K-HEPES, 1 mmol/l K-EGTA, pH 7.4), plus 1 mmol/l $MgCl_2$ and 1 mmol/l ATP (potassium salt). Experiments were digitised into a microcomputer using Pclamp8.0 (Axon Instruments,

La Jolla, CA, USA) software. Off-line analysis was performed using EXCEL (Microsoft).

$^{86}Rb^{+}$ efflux experiments Isolated islets were pre-incubated for 1 h with $^{86}Rb^{+}$ (as rubidium chloride, 1.5 mCi/ml; Amersham Biosciences). Loaded islets were placed in microcentrifuge tubes (30 per group) and washed twice with RPMI-1640 medium (Sigma) at 37°C. $^{86}Rb^{+}$ efflux was assayed by replacing the bathing solution with Ringer's solution with metabolic inhibitor (MI), with or without 1 μmol/l glibenclamide. MI solution contained 2.5 mg/ml oligomycin, 1 mmol/l 2-deoxyglucose, together with 10 mmol/l tetraethylammonium to block voltage-gated K^{+} channels and 10 μmol/l nifedipine to block Ca^{2+} entry. The bathing solution was replaced with fresh solution every 5 min over a 40-min period, and counted in a scintillation counter. $^{86}Rb^{+}$ efflux in the presence or absence of glibenclamide was fitted by a single exponential (see Fig. 1a). The reciprocal of the exponential time constant (rate constant) for each efflux experiment is then proportional to the K^{+} (Rb^{+}) conductance in the islet membranes.

Blood glucose and insulin levels Whole blood was assayed for blood glucose using the glucose dehydrogenase-based enzymatic assay and quantified using a Glucometer Elite Xl meter (Bayer, Elkhart, IN, USA). Plasma insulin was measured using a rat insulin ELISA kit (Crystal Chem, Chicago, IL, USA). Intraperitoneal glucose tolerance tests (GTTs) were done on 12-week-old mice following a 16-h fast. Animals were injected i.p. with glucose (1.0 g/kg body weight).

Insulin-release experiments Following overnight incubation in CMRL (low glucose, 5.6 mmol/l) islets (ten per well in 12-well plates) were pre-incubated in DMEM plus 3 mmol/l glucose for 2 h. The islets were incubated in DMEM plus glucose as indicated for 60 min at 37°C and medium was removed and assayed for insulin content as described above. Experiments were repeated in triplicate. Isolated islets were sonicated on ice prior to estimation of insulin content per islet.

Confocal imaging of microfluidic-device-trapped islets Devices were fabricated using the elastomer polydimethylsiloxane as described [19]. Islets were labelled with 4 μmol/l Fluo-4 (Molecular Probes, Eugene, OR,

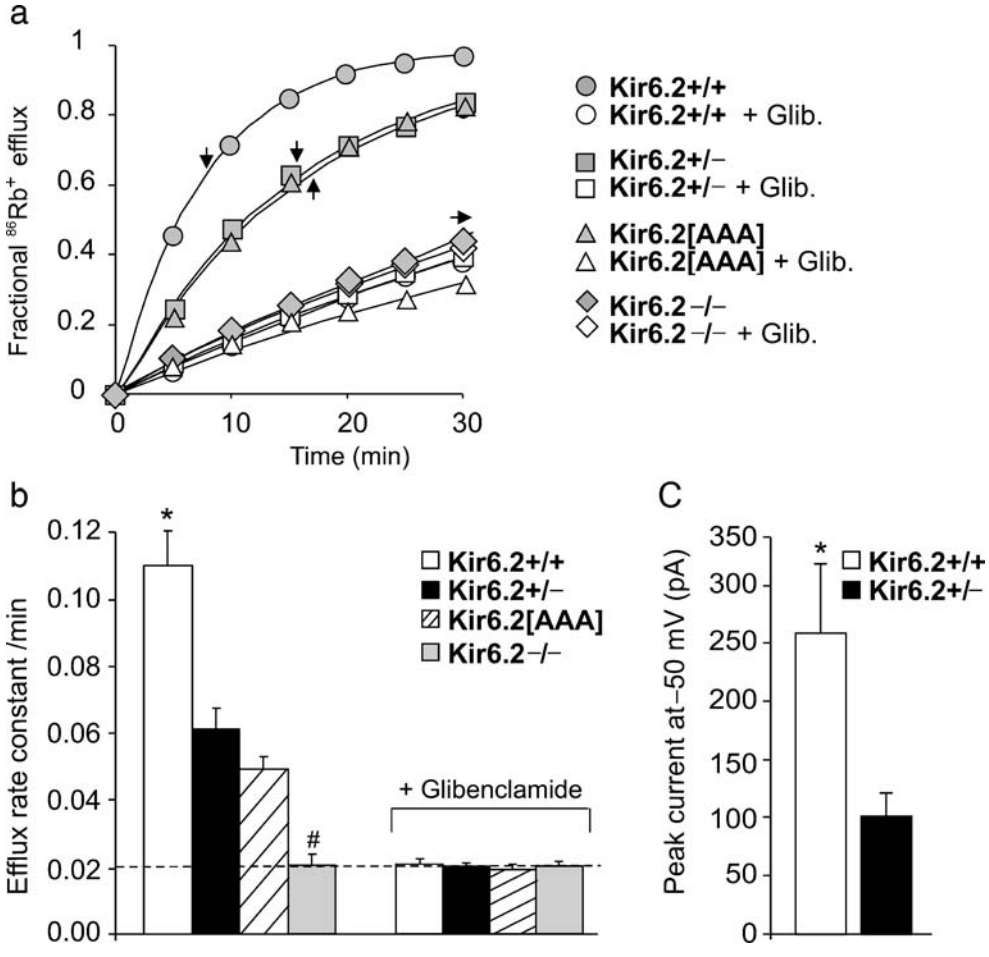

Fig. 1 K_{ATP} channel density is similarly reduced in Kir6.2$^{+/-}$ and Kir6.2[AAA] islets. **a** Fractional $^{86}Rb^{+}$ efflux as a function of time from representative islet samples, in MI (2.5 mg/ml oligomycin plus 1 mmol/l 2-deoxyglucose, 10 mmol/l tetraethylammonium, 10 μmol/l nifedipine with (*filled symbols*) or without (*open symbols*) 1 μmol/l glibenclamide (*Glib.*). *Arrowheads* indicate time constants. **b** Mean $^{86}Rb^{+}$ efflux rate constant for indicated genotypes, in MI (*left*) and in MI plus glibenclamide (*right*) (means±SEM, *n*=5–9 mice, 30 islets in each condition). **c** Peak whole-cell K^{+} current at −50 mV for Kir6.2$^{+/+}$ and Kir6.2$^{+/-}$ pancreatic beta cells (means±SEM, *n*=3 mice, 16 and 15 beta cells, respectively). **p*<0.01 vs all other genotypes, and #*p*<0.01 vs Kir6.2$^{+/-}$ and Kir6.2[AAA]

USA) at room temperature for 2 h in imaging buffer (125 mmol/l NaCl, 5.7 mmol/l KCl, 2.5 mmol/l $CaCl_2$, 1.2 mmol/l $MgCl_2$, 10 mmol/l HEPES, 2 mmol/l glucose and 0.1% BSA, pH 7.4). Imaging was performed using an LSM 510 microscope with a 20×0.75 NA Fluar objective lens (Carl Zeiss, Thornwood, NY, USA). The device was held on the microscope in a humidified temperature-controlled stage (Carl Zeiss) for imaging at 37°C. Fluo-4 was imaged using the 488 nm laser line and the long-pass 505 nm emission filter. Images were collected at 2-s intervals for a period of 2 min after incubation for at least 10 min in 2, 4, 6, 8 or 10 mmol/l glucose.

Image analysis Images were analysed with MetaMorph 5.0 (Universal Imaging Corp., Downington, PA, USA). In order to determine the glucose dependence of intracellular Ca^{2+} oscillations, 'Ca^{2+}-active' cells were identified and outlined while the islets were incubated in 8 mmol/l glucose. Ca^{2+}-active cells (at 8 mmol/l glucose) were defined as cells that exhibited clear, synchronised increase of fluorescence (F/F_0) from baseline, as illustrated in Fig. 5. Then, the same outlined cells (Ca^{2+}-active cells at 8 mmol/l glucose) were analysed for Ca^{2+} oscillations at lower and higher glucose concentrations. Glucose dependence of Ca^{2+} from the whole islet was also measured. The fraction of cells showing synchronised oscillations was then estimated at each glucose concentration (Fig. 5c).

Statistical analysis Data are presented as means±SEM. Differences between samples were compared using the Student's *t*-test or ANOVA and post hoc Duncan's test, as appropriate. A difference was defined as significant when $p<0.05$. Non-significant differences are not indicated in the figures.

Results

Heterozygous Kir6.2$^{+/-}$ and Kir6.2[AAA] islets exhibit a similar reduction in total K_{ATP} channel density Macroscopic K_{ATP} channel density in intact islets was assessed by $^{86}Rb^+$ efflux under metabolic inhibition (MI with oligomycin and 2-deoxyglucose) to lower cellular [ATP]:[ADP] and activate K_{ATP} channels. Figure 1a shows sample fluxes from each of the examined genotypes in the presence and absence of the channel blocker glibenclamide. Fluxes were fitted by single exponentials (time constants are indicated by arrowheads in Fig. 1a), and reciprocal rate constant are plotted in Fig. 1b. In wild-type islets >80% of the flux is glibenclamide-sensitive, indicating a high K_{ATP} conductance, but there is no glibenclamide-sensitive flux in Kir6.2$^{-/-}$ islets. Intermediate fluxes in Kir6.2$^{+/-}$ and Kir6.2[AAA] islets represent ~60% reduction in the glibenclamide-sensitive rate constants, indicating a similar reduction in K_{ATP} conductance. Assuming a simple gene-dosage effect, it would be expected that Kir6.2$^{+/-}$ cells should exhibit a 50% reduction in Kir6.2 protein, but given the requirement of a hetero-octameric stoichiometry for the functional channel complex [9, 10, 20] a greater reduction might be expected. Increasing concentrations of glucose caused similar reductions of $^{86}Rb^+$ efflux rates in both Kir6.2$^{+/+}$ and Kir6.2$^{+/-}$ islets, suggesting similar glucose sensitivity of the underlying K_{ATP} conductances (data not shown).

K_{ATP} channel current in heterozygous Kir6.2$^{+/-}$ and Kir6.2$^{+/+}$ beta cells was also measured using a whole-cell membrane patch-clamp. Maximum resting membrane potential (in zero glucose) was similar (~−75 mV) in both genotypes, but consistent with the $^{86}Rb^+$ flux measurements from intact islets, whole-cell K^+ current peak during whole-cell perfusion with zero ATP was greatly reduced (by

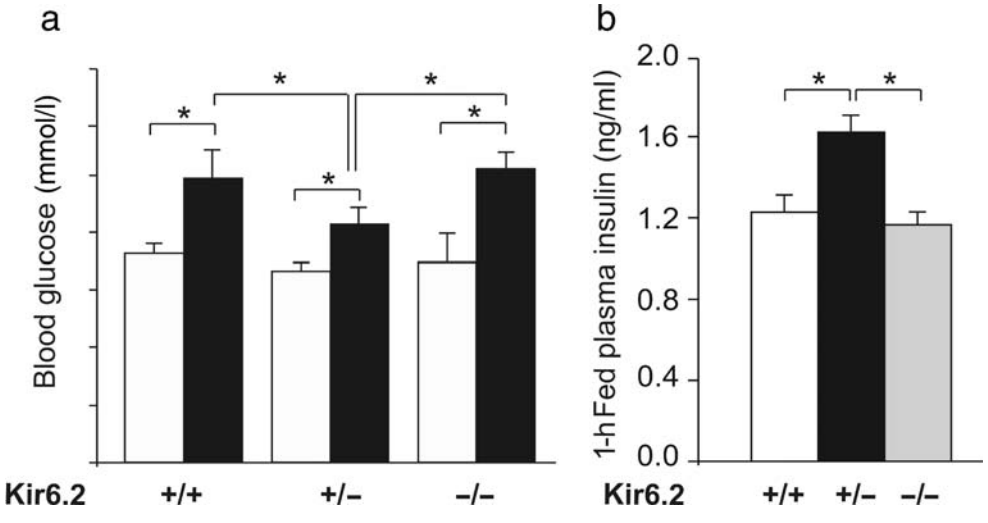

Fig. 2 Kir6.2$^{+/-}$ mice have increased insulin and reduced blood glucose levels after controlled feeding. Blood glucose levels (*open bars*, fasting; *filled bars*, 1-h fed) (**a**) and plasma insulin (**b**) in Kir6.2$^{+/+}$, Kir6.2$^{+/-}$ and Kir6.2$^{-/-}$ mice after 1-h feeding following an overnight fast (16 h) (means± SEM, n=10–16 for glucose and n=5–9 for insulin). *$p<0.01$

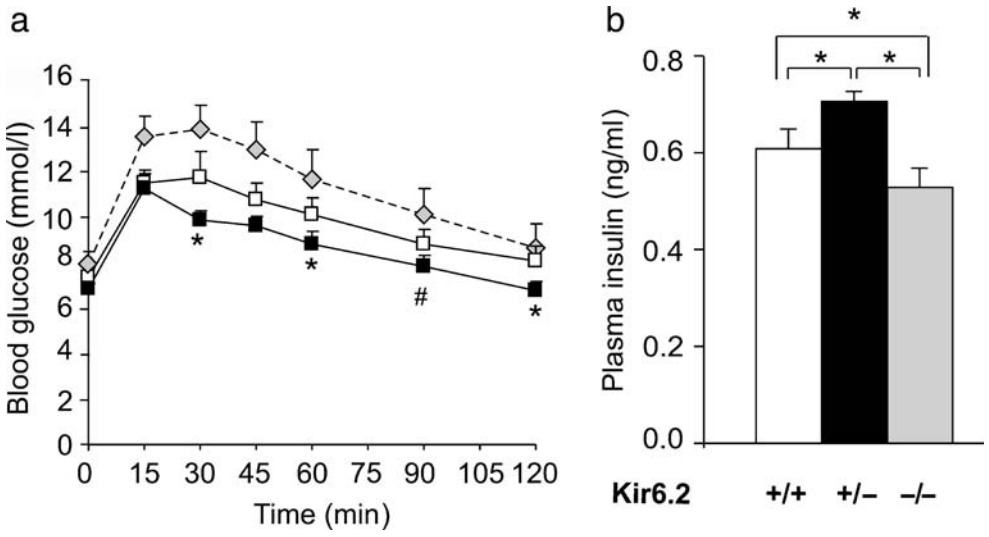

Fig. 3 Kir6.2$^{+/-}$ mice have improved glucose tolerance while Kir6.2$^{-/-}$ mice are mildly glucose-intolerant. GTTs performed on indicated genotypes. Animals were injected i.p. with glucose (1 g/kg) at time t=0. **a** Blood was taken from the tail vein at times indicated and assayed for blood glucose (n=10–16, means± SEM). *White squares*, Kir6.2$^{+/+}$; *black squares*, Kir6.2$^{+/-}$; *grey diamonds*, Kir6.2$^{-/-}$. *p<0.01 vs Kir6.2$^{+/+}$ and Kir6.2$^{-/-}$ and #p<0.01 vs Kir6.2$^{-/-}$ only. **b** Blood samples were taken 30 min after glucose injection and assayed for plasma insulin concentration (n=10–16, means± SEM). *p<0.01

~60%) in Kir6.2$^{+/-}$ beta cells compared with Kir6.2$^{+/+}$ beta cells (Fig. 1c).

Kir6.2$^{+/-}$ mice hypersecrete insulin and have enhanced glucose tolerance, while Kir6.2$^{-/-}$ mice are mildly glucose-intolerant In response to ad libitum feeding for 1 h after overnight fasting, in vivo insulin secretion and blood glucose concentration were examined to test the secretory efficiency of a mixture of nutrient secretagogues. Fasting blood glucose showed no significant differences between all three phenotypes. However, Kir6.2$^{+/-}$ mice maintained significantly lower blood glucose levels after 1-h feeding than Kir6.2$^{+/+}$ and Kir6.2$^{-/-}$ (Fig. 2a). Consistent with a hypersecretory phenotype, Kir6.2$^{+/-}$ plasma insulin was consistently higher than in control Kir6.2$^{+/+}$ or Kir6.2$^{-/-}$ littermates (Fig. 2b).

To characterise the physiological consequence of the elevated circulating insulin in the Kir6.2$^{+/-}$ mice, GTTs were performed (Fig. 3a). Mice were injected i.p. with glucose, following an overnight fast. Kir6.2$^{+/-}$ mice showed enhanced glucose tolerance, with a more rapid normalisation of blood glucose, while Kir6.2$^{-/-}$ mice were mildly glucose-intolerant, compared with wild-type littermates. Although all genotypes displayed similar blood glucose levels prior to glucose infusion, Kir6.2$^{+/-}$ mice exhibited significantly lower blood glucose levels at 30, 60 and 120 min after injection. Plasma insulin, measured at 30 min after glucose infusion, was also higher in Kir6.2$^{+/-}$ mice and lower in Kir6.2$^{-/-}$ mice than in control Kir6.2$^{+/+}$ littermates (Fig. 3b). Thus Kir6.2$^{+/-}$ mice exhibit enhanced glucose tolerance, and elevated circulating insulin, a very similar hyperinsulinaemic phenotype to that previously reported in Kir6.2[AAA] mice [15].

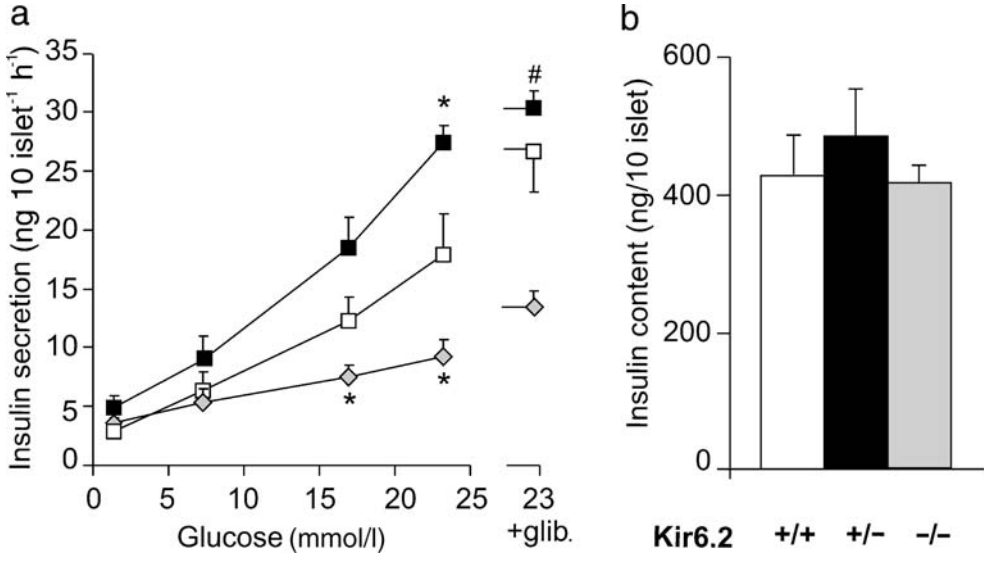

Fig. 4 GSIS is increased in Kir6.2$^{+/-}$ islets but suppressed in Kir6.2$^{-/-}$ islets. **a** Glucose dependence of insulin secretion from islets of indicated genotypes incubated for 1 h at non-stimulatory and stimulatory glucose concentrations (1, 7, 16.7, 23 mmol/l, and 23 mmol/l plus 1 μmol/l glibenclamide [*Glib.*]) (means±SEM, n=8). *White squares*, Kir6.2$^{+/+}$; *black squares*, Kir6.2$^{+/-}$; *grey diamonds*, Kir6.2$^{-/-}$. *p<0.01 vs Kir6.2$^{+/+}$, and #p<0.01 vs Kir6.2$^{-/-}$ only. **b** Insulin content of islets from 4-month-old mice (means±SEM, n=5 mice in each group)

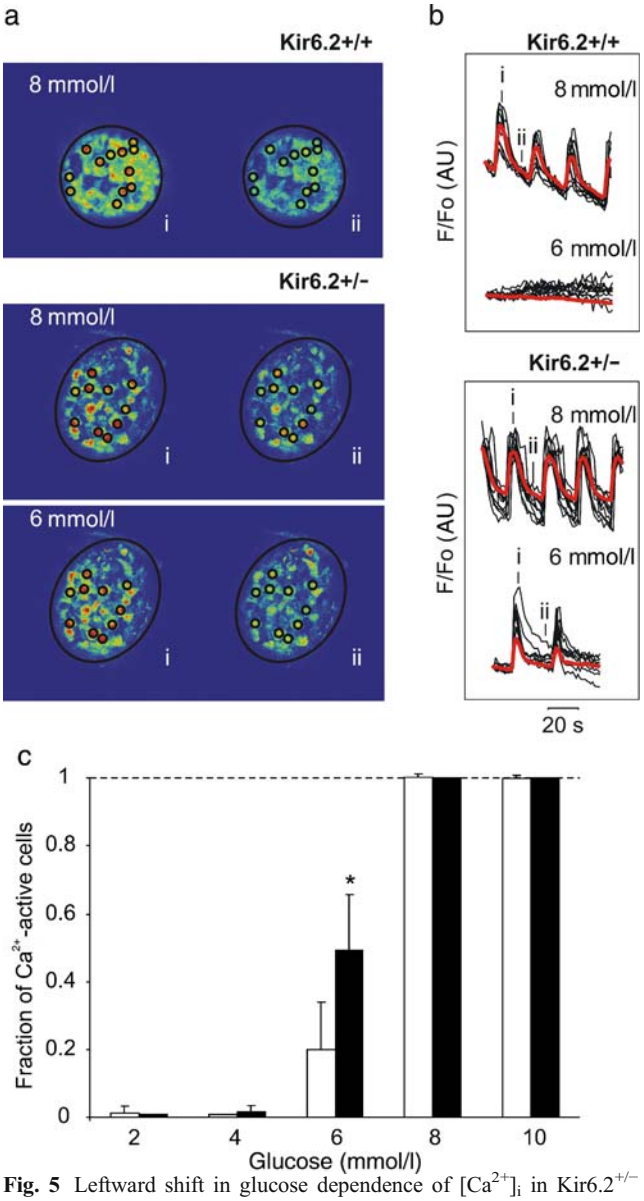

Fig. 5 Leftward shift in glucose dependence of $[Ca^{2+}]_i$ in Kir6.2$^{+/-}$ islets. **a** Representative confocal images of intracellular calcium in Kir6.2$^{+/+}$ (*top panel*) and Kir6.2$^{+/-}$ islets (*bottom two panels*). Individual cells showing synchronised oscillations at 8 mmol/l glucose were *outlined* and frames are depicted when overall Ca^{2+} was high (i, *left of panel*) or low (ii, *right of panel*). Similarly, frames showing synchronously high and low Ca^{2+} at 6 mmol/l glucose are shown for the Kir6.2$^{+/-}$ islet. **b** Relative fluorescence intensity (F/F_0) vs time taken from each outlined cell in **a** (*black traces*) and from the whole islets (*red traces*) in **a** are shown at 6 and 8 mmol/l glucose. The timing of the frames in **a** are indicated (i and ii). **c** Fraction of Ca^{2+}-active cells at different glucose concentrations in Kir6.2$^{+/+}$ (*empty bars*) and Kir6.2$^{+/-}$ (*filled bars*) islets (means±SEM, n=7 islets, 10–12 cells per islet). *p=0.052 vs Kir6.2$^{+/+}$limb

GSIS is increased in Kir6.2$^{+/-}$ islets, but is suppressed in Kir6.2$^{-/-}$ islets GSIS was assessed by incubating Kir6.2$^{+/+}$, Kir6.2$^{+/-}$ and Kir6.2$^{-/-}$ islets at non-stimulatory (1 mmol/l) and stimulatory glucose concentrations (7, 16.7 and 23 mmol/l). Kir6.2$^{+/-}$ islets displayed a marked increase in insulin secretion at stimulatory glucose concentrations (Fig. 4a). This increase in GSIS can account for the significantly elevated serum insulin levels and enhanced glucose tolerance in Kir6.2$^{+/-}$ mice. Conversely, and consistent with impaired glucose tolerance, Kir6.2$^{-/-}$ islets showed a weak secretory response to glucose (Fig. 4a), as described previously [14]. Maximum insulin release was also measured by stimulation with a high glucose concentration (23 mmol/l) plus glibenclamide (1 μmol/l). Similar maximum insulin secretion was obtained in Kir6.2$^{+/+}$ and Kir6.2$^{+/-}$ islets (41.5±1.6 and 44.5±1.5 ng (10 islets)$^{-1}$ h^{-1}, respectively), but it was much lower in Kir6.2$^{-/-}$ islets (16.1±1.1 ng (10 islets)$^{-1}$ h^{-1}).

Islet insulin content was determined in parallel with insulin secretion from non-glucose-challenged islets (50–100 islets in replicates of 10, from each animal). Total insulin content was not significantly different among the genotypes (Fig. 4b).

Leftward shift in glucose dependence of $[Ca^{2+}]_i$ in Kir6.2$^{+/-}$ islets The expected phenotype of reduced K_{ATP} density would be a left-shift in the glucose dependence of electrical excitability and of $[Ca^{2+}]_i$. To assess this directly, we examined the glucose dependence of $[Ca^{2+}]_i$ oscillations in intact isolated islets labelled with Fluo-4, in a microfluidic device that permitted continuous fluid flow and multiple solution changes [21]. Labelled islets were exposed to increasing concentrations of glucose (2–10 mmol/l), for at least 10 min in each concentration. Synchronised Ca^{2+} activity from individual beta cells within the islet was observed in both Kir6.2$^{+/+}$ and Kir6.2$^{+/-}$ islets at 8 mmol/l glucose (Fig. 5a). We therefore tracked whole-islet Ca^{2+} concentration and $[Ca^{2+}]_i$ in individual beta cells (outlined cells in Fig. 5) at 8 mmol/l glucose, then retrospectively analysed the Ca^{2+} activity in the same outlined cells at lower and higher glucose concentration. Figure 5a shows a representative islet from the Kir6.2$^{+/+}$ and Kir6.2$^{+/-}$ genotypes, at a peak (i) and trough (ii) of synchronised oscillations. Figure 5b shows the oscillatory Ca^{2+} behaviour from the individual outlined cells in Fig. 5a, at 6 and 8 mmol/l glucose (black traces, peak [i] and trough [ii] indicated), as well as the average Ca^{2+} from the whole islet (red traces). Consistent with previous studies [21], Kir6.2$^{+/+}$ cells typically showed no oscillatory behaviour at 6 mmol/l glucose (i.e. $[Ca^{2+}]_i$ remained uniformly low) (Fig. 5a,b). By contrast, most Kir6.2$^{+/-}$ islets showed Ca^{2+} oscillations at 6 mmol/l glucose (Fig. 5a,b). The fraction of Ca^{2+}-active cells is shown in Fig. 5c. At lower glucose concentrations (2 and 4 mmol/l) the fraction of Ca^{2+}-active cells was negligible in both genotypes (Fig. 5c). The fraction of Ca^{2+}-active cells was higher in Kir6.2$^{+/-}$ than Kir6.2$^{+/+}$ islets at 6 mmol/l glucose. Although not statistically significant (p=0.052), this leftward-shift of excitability in Kir6.2$^{+/-}$ islets is consistent with, and probably underlies the left-

ward-shift in glucose-dependent insulin secretion in Kir6.2$^{+/-}$ islets (Fig. 4).

Heterozygous SUR1$^{+/-}$ islets reiterate reduced K_{ATP} channel density, insulin hypersecretion, and mildly enhanced glucose tolerance Given the strict stoichiometry between SUR1 and Kir6.2 in the generation of K_{ATP} channels, it is expected that loss of either subunit should have similar effects on K_{ATP} channel density. As shown in Fig. 6a,b, there is no glibenclamide-sensitive $^{86}Rb^{+}$ flux in SUR1$^{-/-}$ islets. Intermediate fluxes in SUR1$^{+/-}$ islets represent ~60% reduction in the glibenclamide-sensitive rate constants, again indicating a similar reduction in K_{ATP} conductance to that seen in heterozygous Kir6.2$^{+/-}$ islets (Fig. 1).

We examined the whole-animal consequences of this reduced beta cell K_{ATP} conductance. Although fasted blood glucose was not different between wild-type and SUR1$^{+/-}$ littermates (Fig. 6e), fed blood glucose was slightly lower (Fig. 6f), as seen in Kir6.2$^{+/-}$ mice. Similarly, SUR1$^{+/-}$ mice showed slightly enhanced glucose tolerance compared with littermate controls, whereas SUR1$^{-/-}$ mice were extremely glucose-intolerant (Fig. 6c). Consistent with the differential whole-animal phenotypes, isolated SUR1$^{+/-}$ islets also displayed increased GSIS (Fig. 6d). Maximum insulin release (in 23 mmol/l glucose plus glibenclamide) was again similar in SUR1$^{+/+}$ and SUR1$^{+/-}$ islets and much lower in SUR1$^{-/-}$ islets (Fig. 6d).

Discussion

Decreased K_{ATP} channel activity and insulin secretion in vivo: predictions vs findings It is now clear that K_{ATP} channels are a critical link between glucose metabolism and insulin secretion, underlined by the finding that gain of K_{ATP} activity can cause permanent neonatal diabetes mellitus (PNDM) [22–25] whereas CHI results from loss-of-function K_{ATP} mutations [5, 26–30]. We previously described a mouse model of beta cell K_{ATP} gain-of-function that reiterates the PNDM phenotype [31]. However, mice completely lacking K_{ATP} channels (by knocking out either the SUR1 or Kir6.2 subunit) do not completely reiterate the CHI phenotype. In K_{ATP} channel KO mice, hypersecretion reportedly occurs immediately after birth, but rapidly progresses to a relative undersecretion [12–14, 17]. K_{ATP} channels are distributed throughout the body and, conceivably, lack of K_{ATP} in other tissues contributes to the hyperinsulinaemia and secondary progression in Kir6.2$^{-/-}$ or SUR1$^{-/-}$ animals [32–35]. Lack of K_{ATP} in skeletal muscle causes enhanced basal and insulin-stimulated glucose uptake [32]. Activation of K_{ATP} channels in the mediobasal hypothalamus can lower blood glucose through inhibition of hepatic gluconeogenesis and SUR1$^{-/-}$ mice are resistant to this inhibitory action [35]. It is important to bear these complex positive and negative effects of K_{ATP} KO on lowering of blood glucose in mind, but at this point it is unclear how they will play out in terms of the whole-animal progression.

In terms of insulin secretory phenotype, recent papers on SUR1 KO mice have shown somewhat contradictory results, even demonstrating a basal insulin hypersecretion and maintained insulin secretion at high glucose concentrations [36, 37]. A full explanation for the complexity of the results is yet to be achieved, although there is clear evidence that glutamine and other amino acids can potently stimulate secretion in these SUR1 KO islets [37, 38]. These mice also exhibit near-normal insulin secretion in response to feeding, which could account for the euglycaemia [12]. Nevertheless, in terms of modelling CHI, it is clear that mice with a complete absence of beta cell K_{ATP} channels are glucose-intolerant and neither persistently hyperinsulinaemic nor hypoglycaemic.

Conversely, mice expressing a dominant-negative Kir6.2[AAA] transgene in beta cells show an incomplete reduction of K_{ATP} channel activity (~70%) and demonstrate both an enhanced glucose stimulation of insulin secretion and hyperinsulinism that persists through adulthood [15]. As discussed below, we suggest that genetic suppression of K_{ATP} activity leads to enhanced excitability and insulin secretion, but with potentially different long-term consequences depending on severity of the suppression: incomplete loss of K_{ATP} (e.g. Kir6.2[AAA] mice) causes a maintained hyperinsulinism whereas complete loss may transiently cause hypersecretion, followed by a secretory deficit and reduced glucose tolerance. In the present study, heterozygous Kir6.2$^{+/-}$ or SUR1$^{+/-}$ mice are both shown to have a significant (~60%) reduction of K_{ATP} channel activity in islets, presumably distributed uniformly among all beta cells. Consistent with the proposed hypothesis, these mice demonstrate a similar hyperinsulinaemic phenotype to Kir6.2[AAA] mice. Whereas there is a clear insulin undersecretion from SUR1$^{-/-}$ islets [36] and glucose insensitivity in Kir6.2$^{-/-}$ mice [14], partial loss of K_{ATP} in Kir6.2$^{+/-}$ and SUR1$^{+/-}$ mice leads to an insulin hypersecretion that fails to progress to undersecretion. Quantitatively, the phenotypes are not identical, clearly SUR1$^{-/-}$ mice are even more glucose-intolerant than Kir6.2$^{-/-}$ mice (Figs. 3 and 6), and enhanced glucose tolerance is more pronounced in Kir6.2$^{+/-}$ than SUR1$^{+/-}$ mice (Figs. 3 and 6). In part these differences may be the result of strain differences: we did not perform the multiple backcrosses of the SUR1$^{-/-}$ mice necessary to reach

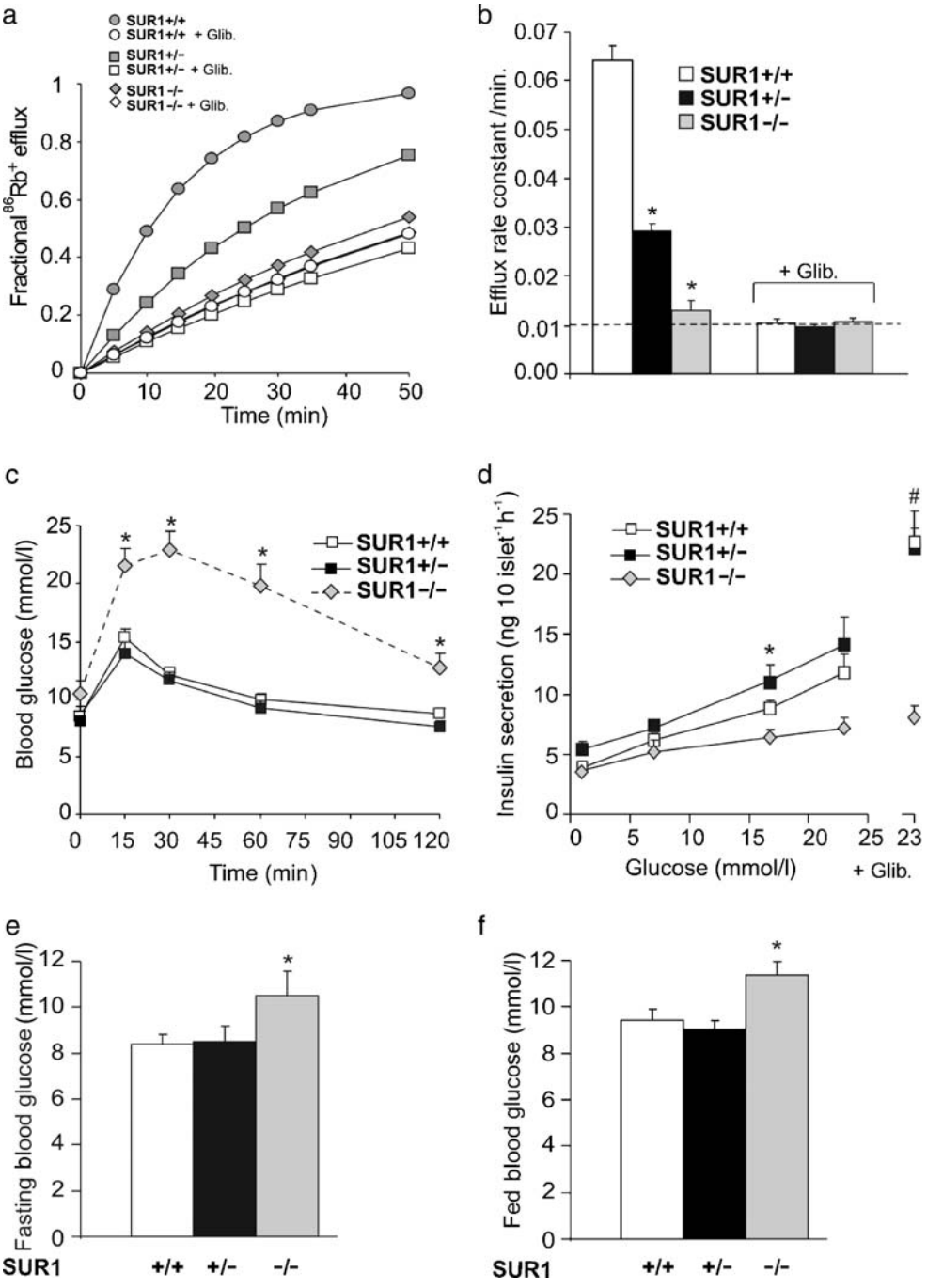

Fig. 6 $SUR1^{+/-}$ mice exhibit reduced beta cell K_{ATP} and enhanced GSIS. **a** Fractional $^{86}Rb^{+}$ efflux as a function of time from representative islet samples in MI (2.5 mg/ml oligomycin plus 1 mmol/l 2-deoxyglucose, 10 mmol/l tetraethylammonium, 10 μmol/l nifedipine, initiated at time t=0), with (*filled symbols)* or without (*open symbols*) 1 μmol/l glibenclamide (*Glib.*). **b** Mean $^{86}Rb^{+}$ efflux rate constant for indicated genotypes, in MI (*left*) and in MI plus glibenclamide (*right*) (means±SEM, n=4–8 mice, 30 islets in each condition). **c** GTTs performed on indicated genotypes. Animals were injected i.p. with glucose (1 g/kg) at time t=0. Blood was taken from the tail vein at times indicated and assayed for blood glucose (means±SEM, n=8–12, *p<0.01 vs $SUR1^{+/+}$). **d** Glucose dependence of insulin secretion from islets of indicated genotypes incubated for 1 h at non-stimulatory and stimulatory glucose concentrations (1, 7, 16.7, 23 mmol/l, and 23 mmol/l plus 1 μmol/l glibenclamide [*Glib.*]) (means±SEM, n=8). *p<0.01 vs $SUR1^{+/+}$, and $^{\#}p$<0.01 vs $SUR1^{-/-}$. **e**, **f** Blood glucose evels in fed (**e**) or overnight fasted (**f**) littermate $SUR1^{+/+}$, $SUR1^{+/-}$ and $SUR1^{-/-}$ mice (means±SEM, n=8–12 animals). *Significant differences, p<0.01

isogenicity. In addition, K_{ATP} channels in skeletal muscle are formed from Kir6.2 plus SUR2A [32, 39], which will mean that Kir6.2 KO mice (but not SUR1 KO mice) will have increased peripheral glucose sensitivity, which may contribute to maintenance of glucose tolerance.

Beta cell hyperexcitability and secretory phenotype: an 'inverse-U' model We have thus further proposed [18], and the data here support, an inverse-U model [40] for the general beta cell response to hyperexcitability generated by alterations of K^{+} (or other) conductances (Fig. 7). The essence of the model is that as excitability increases, there is an expected hypersecretory response to glucose, but as excitability is increased above a threshold, either developmentally (e.g. in K_{ATP} KO animals [12–14]) or in response to altered stimulatory conditions (e.g. high-fat diet [18]), a secondary loss of secretory capacity leads to relative hypoinsulinaemia and glucose intolerance. Graded degrees of hyperexcitability will lead to graded enhancements of insulin secretion until, above some threshold, beta cells are driven to insulin secretory failure. At this juncture, we can only speculate on the trigger behind the secondary secretory

failure. Elevated $[Ca^{2+}]_i$ [14, 15], as a downstream consequence of reduced K_{ATP} channel activity, is an obvious candidate to underlie hyperinsulinaemia. However, by apoptotic or other regulatory mechanisms, Ca^{2+} could also lead to secretory failure at even higher levels.

We have previously demonstrated in Kir6.2[AAA] islets [41], and now in Kir6.2$^{+/-}$ islets (Fig. 5), a leftward shift in glucose dependence of $[Ca^{2+}]_i$ oscillations in Kir6.2$^{+/-}$ islets that is consistent with the increased insulin secretion. Adult Kir6.2$^{+/-}$, SUR1$^{+/-}$ and Kir6.2[AAA] mice, which have a similar decrease in K_{ATP} channel activity and hyperinsulinaemia [15], would thus be positioned on the 'ascending' limb of the inverse-U progression (Fig. 7). Conversely, Kir6.2$^{-/-}$ or SUR1$^{-/-}$ mice, which have maximal hyperexcitability, and high $[Ca^{2+}]_i$ even at low glucose concentration [14, 42], would be positioned on the 'descending' limb (Fig. 7). It also predicted that an increase of K_{ATP} density, or loss of sensitivity to inhibitory glucose, would lead to reduced islet excitability and secretory response. Transgenic mice expressing mutant beta cell K_{ATP} channels (Kir6.2[ΔN30]) with reduced ATP sensitivity (and presumably reduced glucose sensitivity) do have a severely undersecreting phenotype [31], and this appears to be the mechanism of PNDM in humans [22, 23, 43]. We can thus add an extension of the 'ascending' limb into the region of subnormal excitability (Fig. 7).

Potential relevance to CHI and type 2 diabetes That three distinct animal models of reduced beta cell K_{ATP} density (Kir6.2$^{+/-}$, SUR1$^{+/-}$ and Kir6.2[AAA] [15]) exhibit hyperinsulinism, whereas three models of complete loss of K_{ATP} (Kir6.2$^{-/-}$, SUR1$^{-/-}$ and Kir6.2[G132S] [12–14]) are glucose-intolerant, may have considerable relevance both to the secondary progression of CHI and to the progression from prediabetic phenotype to glucose intolerance and type 2 diabetes in humans.

Heterozygous loss-of-function mutations of beta cell K_{ATP} subunits (SUR1, encoded by *ABCC8*, or Kir6.2, encoded by *KCNJ11*) underlie CHI [5–7, 29, 30, 44]. While mice with reduced beta cell K_{ATP} reiterate a hyperinsulinaemic phenotype (this study and [15]), mice completely lacking beta cell K_{ATP} activity unexpectedly show glucose intolerance and loss of GSIS as adults [12–14]. One implication of these findings is that persistent hyperinsulinism in humans might reflect incomplete loss of K_{ATP}. The phenotype of many CHI mutations in recombinant expression [5, 30, 44] would actually suggest that a reduced, but not complete, absence of K_{ATP} channels [45] is likely. Consistent with this idea, Henwood et al. [46] have recently demonstrated that some CHI patients with K_{ATP} channel mutations must maintain some K_{ATP} channel activity, since the patients were responsive to the K_{ATP} channel drugs tolbutamide and diazoxide. Although carriers of loss-of-function (but not complete KO) SUR1 mutations have reportedly normal glucose tolerance and insulin secretion [47], we may suggest that heterozygous carriers of complete KO mutations might have enhanced glucose tolerance and subclinical hyperinsulinism. Interestingly, one of the early studies of human CHI mutations reported a homozygous Kir6.2 truncation mutation in an affected patient [6], but unfortunately, there were no clinical data on the heterozygous parents.

A second implication is that in humans with severe loss of K_{ATP}, a progression from hyperinsulinaemia to glucose intolerance might be expected, as seen in K_{ATP} KO mice [12–15]. Although only limited data are available, there are reports that some CHI patients, even those non-surgically treated, can spontaneously progress to diabetes [7, 48, 49]. In addition, we have shown that normally hypersecreting Kir6.2[AAA] transgenic mice on a Kir6.2$^{+/-}$ background (which, although untested, presumably have a greater reduction in K_{ATP} channel activity than each genotype alone), but not Kir6.2$^{+/-}$ mice, can progress from a hypersecreting phenotype to an undersecreting diabetic phenotype, when challenged by a high-fat diet [18]. We may thus speculate that any mechanism causing hyperexcitability of islets, for example by decreasing K_{ATP} channel density or activity, will cause an initially hypersecreting phenotype. However, further increase in excitability, above some threshold, can progress to an undersecretory diabetic phenotype. Such a progression has potential parallels to the typical progres-

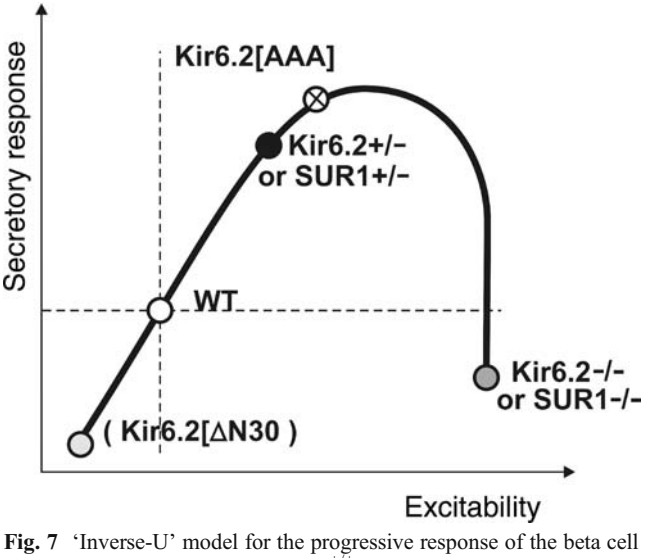

Fig. 7 'Inverse-U' model for the progressive response of the beta cell to increasing excitability. Kir6.2$^{+/+}$ islets represent the normal secretory responsiveness to glucose. Both Kir6.2[AAA] (~60% decrease of K_{ATP} [15]) and Kir6.2$^{+/-}$ islets (~60% reduction in K_{ATP} channel density, present work) show a similar hyperinsulinaemic phenotype, placing these two independent mouse models of decreased K_{ATP} channel activity on the 'ascending' limb. Kir6.2$^{-/-}$ islets with maximally enhanced excitability (100% decrease of K_{ATP}) show an undersecretory phenotype as adults [12–14], placing them on the 'descending' limb

sion of type 2 diabetes—from compensatory insulin hypersecretion to beta cell failure.

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