Original Article

Mutations at the Same Residue (R50) of Kir6.2 (*KCNJ11*) That Cause Neonatal Diabetes Produce Different Functional Effects

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Heterozygous mutations in the human Kir6.2 gene (*KCNJ11*), the pore-forming subunit of the ATP-sensitive K^+ channel (K_{ATP} channel), are a common cause of neonatal diabetes. We identified a novel *KCNJ11* mutation, R50Q, that causes permanent neonatal diabetes (PNDM) without neurological problems. We investigated the functional effects this mutation and another at the same residue (R50P) that led to PNDM in association with developmental delay. Wild-type or mutant Kir6.2/SUR1 channels were examined by heterologous expression in *Xenopus* oocytes. Both mutations increased resting whole-cell currents through homomeric and heterozygous K_{ATP} channels by reducing channel inhibition by ATP, an effect that was larger in the presence of Mg^{2+}. However the magnitude of the reduction in ATP sensitivity (and the increase in the whole-cell current) was substantially larger for the R50P mutation. This is consistent with the more severe phenotype. Single–R50P channel kinetics (in the absence of ATP) did not differ from wild type, indicating that the mutation primarily affects ATP binding and/or transduction. This supports the idea that R50 lies in the ATP-binding site of Kir6.2. The sulfonylurea tolbutamide blocked heterozygous R50Q (89%) and R50P (84%) channels only slightly less than wild-type channels (98%), suggesting that sulfonylurea therapy may be of benefit for patients with either mutation. *Diabetes* 55:1705–1712, 2006

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$[ATP]_i$, intracellular ATP concentration; hetR50P, heterozygous R50P; hetR50Q, heterozygous R50Q; homR50P, homomeric R50P; homR50Q, homomeric R50Q; IC_{50}, half-maximal inhibitory concentration; K_{ATP} channel, ATP-sensitive K^+ channel; PNDM, permanent neonatal diabetes; SUR, sulfonylurea receptor.

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Approximately 50% of cases of permanent neonatal diabetes (PNDM) result from heterozygous mutations in *KCNJ11*, the gene encoding Kir6.2, which constitutes the pore-forming subunit of the ATP-sensitive K^+ channel (K_{ATP} channel) (1–11). Some of these mutations also give rise to muscle weakness and developmental delay (intermediate DEND syndrome) or to a severe condition in which neonatal diabetes is associated with developmental delay, muscle weakness, and epilepsy (DEND syndrome) (2).

Kir6.2 serves as the pore-forming subunit of the K_{ATP} channel in multiple tissues (12,13), with four Kir6.2 subunits coming together to form a tetrameric pore through which K^+ ions move (14). Each Kir6.2 subunit is coupled to a regulatory sulfonylurea receptor (SUR) subunit. There are several isoforms of SUR: SUR1 is found in pancreatic β-cells and brain (15), SUR2A in cardiac and skeletal muscle (16), and SUR2B in vascular smooth muscle (17).

K_{ATP} channels couple-cell metabolism to membrane electrical activity by regulating K^+ fluxes across the plasma membrane. They are involved in multiple physiological processes, including glucose homeostasis, protection against ischemic stress in heart and brain, regulation of neuronal electrical activity, and control of vascular tone (18). In general, opening of K_{ATP} channels inhibits electrical activity and leads to cell quiescence, whereas closing of K_{ATP} channels stimulates electrical activity and cellular responses. For example, in pancreatic β-cells, K_{ATP} channels are open at substimulatory glucose concentrations (18–20). As a result, K^+ efflux through open channels holds the β-cell membrane at a hyperpolarized potential, preventing electrical activity and insulin secretion. In contrast, a rise in plasma glucose stimulates glucose uptake and metabolism, causing an increase in ATP and an accompanying decrease in MgADP. These changes in adenine nucleotide concentration produce K_{ATP} channel closure and, as a consequence, membrane depolarization, opening of voltage-gated Ca^{2+} channels, Ca^{2+} influx, and insulin release (18–20).

Both Kir6.2 and SUR subunits are involved in the metabolic regulation of K_{ATP} channel activity. Binding of ATP or ADP to Kir6.2 produces channel inhibition (21), whereas interaction of Mg nucleotides (MgATP and MgADP) with the nucleotide-binding domains of SUR stimulates channel activity (22–24). Thus in the absence of Mg^{2+}, ATP produces K_{ATP} channel inhibition (via Kir6.2),

whereas in the presence of MgATP, K_{ATP} channel activity is determined by the balance between channel inhibition mediated by Kir6.2 and channel activation mediated via SUR.

K_{ATP} channels are the target for sulfonylurea drugs, such as tolbutamide and glibenclamide, which are widely used to treat type 2 diabetes (25). These drugs stimulate insulin secretion by binding to SUR1 and closing β-cell K_{ATP} channels directly, thus bypassing cell metabolism. They have proved effective in treating neonatal diabetes that results from gain-of-function mutations in Kir6.2 (3,5,9–11).

All *KCNJ11* mutations studied to date enhance K_{ATP} channel activity by reducing the inhibitory effect of MgATP. This is achieved in two ways: a reduced inhibitory action of ATP on Kir6.2 (3,4,7,26–29) and an increase in the stimulatory effects of Mg^{2+} nucleotides mediated via SUR1 (28,29). In general, mutations that cause neonatal diabetes alone decrease the inhibitory effect of ATP on Kir6.2 by impairing ATP binding and/or the transduction of ATP binding into closure of the Kir6.2 pore. Mutations that cause DEND syndrome act indirectly, by stabilizing the open state of the channel (26–28), which thereby decreases ATP block (30,31). The precise mechanism by which Kir6.2 mutations enhance the ability of Mg-nucleotide interactions with SUR1 to stimulate K_{ATP} channel activity is still unclear.

Here, we identify a novel heterozygous *KNCJ11* mutation (R50Q), which lies within the predicted ATP-binding site (Fig. 1). We analyze the functional effects of this mutation, which causes only neonatal diabetes, and of a second mutation at the same residue, R50P, which is associated with neonatal diabetes plus additional clinical problems. We show that both mutations decrease K_{ATP} channel inhibition by ATP and enhance activation by Mg^{2+} nucleotides, which leads to an increase in the K_{ATP} current. The extent of this increase is correlated with the severity of the clinical phenotype. Our results also suggest that sulfonylurea therapy may be effective for patients with either of these mutations.

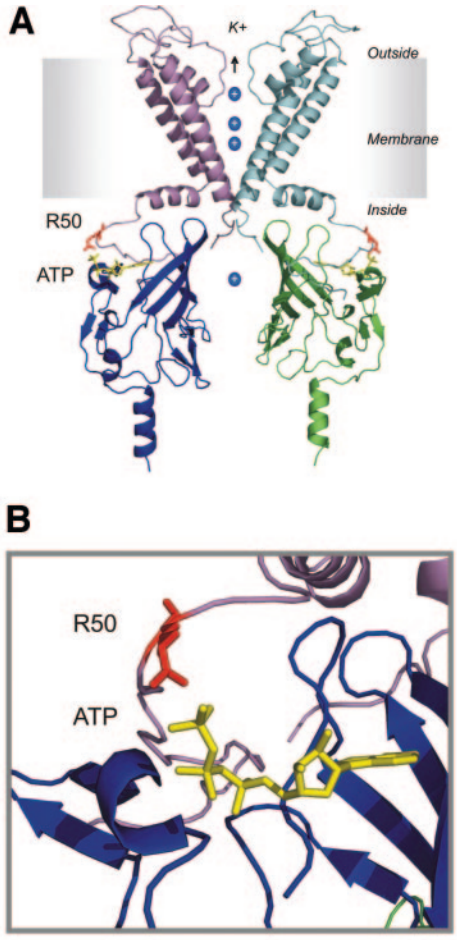

FIG. 1. Location of R50 in Kir6.2. *A*: Molecular model of Kir6.2, viewed from the side. For clarity, each subunit is shown in a different color, and only two transmembrane domains and two cytosolic domains are illustrated. R50P is in ball-and-stick format, and ATP is in yellow. *B*: Molecular model of the ATP-binding site, showing the position of R50. The NH_2 and COOH domains of adjacent subunits are shown in different colors. ATP (yellow) is shown docked into its binding site.

RESEARCH DESIGN AND METHODS

Mutation detection. Genomic DNA was extracted from peripheral lymphocytes using standard procedures. The single exon of the *KCNJ11* gene was amplified in three fragments by PCR. Primer sequences for fragments 1–3 are as described previously (11). Sequencing was performed in both directions using universal M13 primers and a Big Dye Terminator Cycler Sequencing kit v1.1 (Applied Biosystems, Warrington, U.K.) according to manufacturer's instructions. Reactions were analyzed on an ABI 3100 Capillary sequencer (Applied Biosystems).

Molecular biology and oocyte preparation. Human Kir6.2 (GenBank no. NM000525; E23 and I377) and rat SUR1 (GenBank no. L40624) were used in this study. Site-directed mutagenesis of Kir6.2, synthesis of capped mRNA, and preparation and injection of *Xenopus laevis* were performed as previously reported (32). Oocytes were coinjected with ~2 ng SUR1 mRNA and ~0.1 ng wild-type or mutant Kir6.2 mRNA. For each batch of oocytes, all mutations were injected to enable direct comparison of their effects. Oocytes were maintained in Barth's solution and studied 1–7 days after injection (32).

To simulate the heterozygous state, SUR1 was coexpressed with a 1:1 mixture of wild-type and mutant Kir6.2. A potential problem with this approach is that expression levels may differ between wild-type and mutant proteins. However, other approaches are prone to different errors, and simulating the heterozygous state by coinjection of wild-type and mutant subunits is probably least prone to error (28). Furthermore, coexpression of two mRNAs most closely simulates the situation in the patient's cells (where differences in expression may also occur).

Electrophysiology. Whole-cell currents were recorded using a two-electrode voltage clamp (32) in response to voltage steps of ±20 mV from a holding potential of −10 mV, in a solution containing 90 mmol/l KCl, 1 mmol/l $MgCl_2$, 1.8 mmol/l $CaCl_2$, and 5 mmol/l HEPES (pH 7.4 with KOH). Metabolic inhibition was induced by 3 mmol/l sodium azide, and 0.5 mmol/l tolbutamide used to block K_{ATP} channels, as indicated. Macroscopic currents were recorded from giant inside-out patches (32). The pipette solution contained 140 mmol/l KCl, 1.2 mmol/l $MgCl_2$, 2.6 mmol/l $CaCl_2$, and 10 mmol/l HEPES (pH 7.4 with KOH). The Mg-free internal (bath) solution contained 107 mmol/l KCl, 1 mmol/l K_2SO_4, 10 mmol/l EGTA, 10 mmol/l HEPES (pH 7.2 with KOH), and K_2ATP, as indicated. Mg-containing solution was made by adding 2 mmol/l $MgCl_2$ to Mg-free solution and using MgATP rather than ATP.

The macroscopic slope conductance was measured between −20 and −100 mV, in response to 3-s voltage ramps from −100 to +110 mV (holding potential −10 mV). To control for possible rundown, G_c was taken as the mean of the conductance in control solution before and after ATP application. ATP concentration-response curves were fit with the Hill equation:

$$G/G_c = 1/[1 + ([ATP]/IC_{50})^h] \quad (1)$$

where [ATP] is the ATP concentration, IC_{50} is the [ATP] at which inhibition is half-maximal, and h is the slope factor (Hill coefficient). In the case of R50P channels studied in the presence of Mg^{2+}, the current was not completely blocked even at high [ATP] and Eq. 1 was modified to

$$G/G_c = a + (1 - a)/[1 + ([ATP]/IC_{50})^h] \quad (2)$$

where a represents the fraction of unblocked current at saturating [ATP].

Single-channel currents were recorded at −60 mV from inside-out patches, as described previously (30). Because homomeric R50P (homR50P) channels ran down rapidly after patch excision, single-channel currents were measured after channel activity had reached a steady state, and wild-type channels were studied at the same time point. Open probability was determined from current

records of ~1-min duration as I/iN, where I is the macroscopic current, i is the single-channel current amplitude, and N is the number of channels. Data are means ± SE.

RESULTS

Patient details. The first patient (who carries the R50P mutation) has been partially described previously (nd-TO/2 [8]). He was born at 40 weeks gestation weighing 2,730 g. He presented at 3 months with a plasma glucose of 62 mmol/l and features of both diabetic hyperosmolar coma osmolality (405 milliosmoles, plasma Na^+ 162 mmol/l) and diabetic ketoacidosis (blood pH 7.1; HCO_3^- 8 mEq/l). Metabolic abnormalities were initially treated with fluids and insulin, and after 24 h, osmolarity had fallen to 329 mOsm. However the patient did not regain consciousness for 6 days, at which time hypertonic seizures of all limbs were observed. An electroencephalogram showed diffusely abnormal electrical activity. The patient has marked motor and intellectual delay and was unable to walk unaided until 4.5 years. He has continued to have generalized seizures, but these are well controlled with sodium valproate. A magnetic resonance imaging scan showed extended areas of gliosis of the white matter. The patient was originally treated with insulin (0.71 unit · kg^{-1} · day^{-1}), but that treatment was subsequently replaced with glibenclamide at a dose (0.6 mg · kg^{-1} · d^{-1}) that is the highest among nine Italian patients with KCNJ11-PNDM currently transferred from insulin to sulfonylureas.

Patient 2 carries a novel mutation at R50, R50Q. She is a Polish female born to two nondiabetic European-Caucasian parents. She weighed 3,000 g and was born at 40 weeks gestation. She was diagnosed with diabetes on the basis of polyuria, thirst, and failure to put on weight at 26 weeks. At this time, she had a random glucose blood glucose level of 33 mmol/l. She was immediately treated with insulin and has remained on this treatment. At 7 years of age, she had good glycemic control on 2 units · kg^{-1} · day^{-1} insulin with an HbA_{1c} that ranged between 6 and 7%. She has not shown any evidence of neurological abnormalities or mental delay during routine pediatric examinations, her motor development was consistent with her age, and she has not had any seizures.

Molecular genetic analysis. Patient 1 has already been reported to have the R50P mutation (8). Patient 2 was found to have a novel R50Q mutation. The arginine at residue 50 is conserved across other species, and the R50Q variant was not found in more than 200 normal chromosomes. Neither parent carried the R50Q variant, and microsatellite analysis confirmed family relationships, proving that it was a de novo mutation.

Effects of R50 mutations on whole-cell K_{ATP} channel currents. When expressed in *Xenopus* oocytes, Kir6.2/SUR1 channels are normally closed because of the high intracellular ATP concentration ($[ATP]_i$), and they open only in response to metabolic inhibitors, such as azide, which lower $[ATP]_i$ (Fig. 2). In contrast, substantial resting K^+ currents were present in oocytes expressing either homR50P or homomeric R50Q (homR50Q) channels (Fig. 2). These currents were further increased by azide, suggesting that the channels are not fully open at the resting $[ATP]_i$ of the oocyte. They were also substantially blocked by 0.5 mmol/l tolbutamide, a concentration that fully saturates the high-affinity binding site for sulfonylureas (25,33). However, the efficacy of block was significantly less for homR50P channels (Fig. 2).

Because all patients are heterozygotes, their β-cells will contain a mixture of wild-type and mutant Kir6.2. We therefore explored the functional effects of Kir6.2 mutations on K_{ATP} channel function in the simulated heterozygous state, by coinjecting a 1:1 mixture of mutant and wild-type Kir6.2 together with SUR1. This will produce a mixed population of homomeric wild-type channels, homomeric mutant channels, and heteromeric channels containing between one and three mutant subunits. We refer

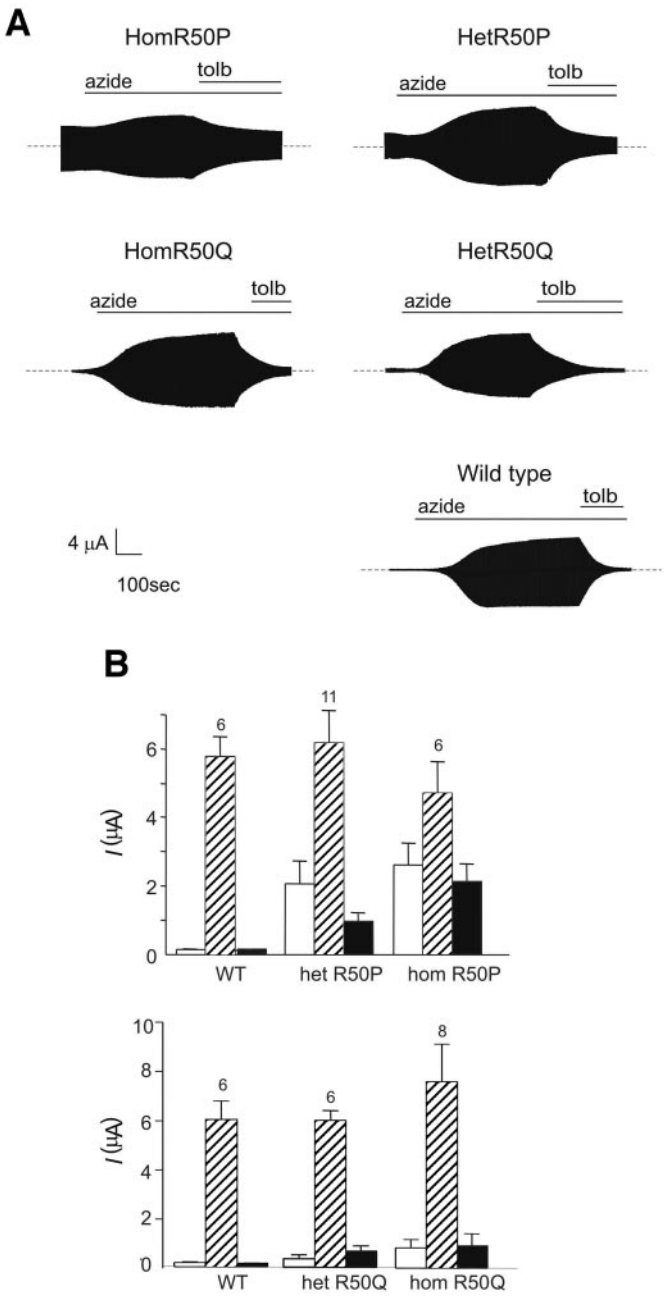

FIG. 2. Comparison of whole-cell currents of wild-type and mutant K_{ATP} channels. *A*: Whole-cell currents recorded from *Xenopus* oocytes coexpressing SUR1 and Kir6.2, Kir6.2-R50P (homR50P), Kir6.2-R50Q (homR50P), both Kir6.2 and Kir6.2-R50Q (hetR50Q), or both Kir6.2 and Kir6.2-R50P (hetR50P) in response to voltage steps of ±20 mV from a holding potential of −10 mV. Bars indicate the times of application of 3 mmol/l azide and 0.5 mmol/l tolbutamide (tolb). *B*: Mean steady-state whole-cell K_{ATP} channel currents (as indicated) evoked by a voltage step from −10 to −30 mV before (□) and after application of 3 mmol/l azide (▨) and in the presence of 3 mmol/l azide plus 0.5 mmol/l tolbutamide (■). The number of oocytes is given above the bars. *Top*, R50P channels. *Bottom*, R50Q channels WT, wild type.

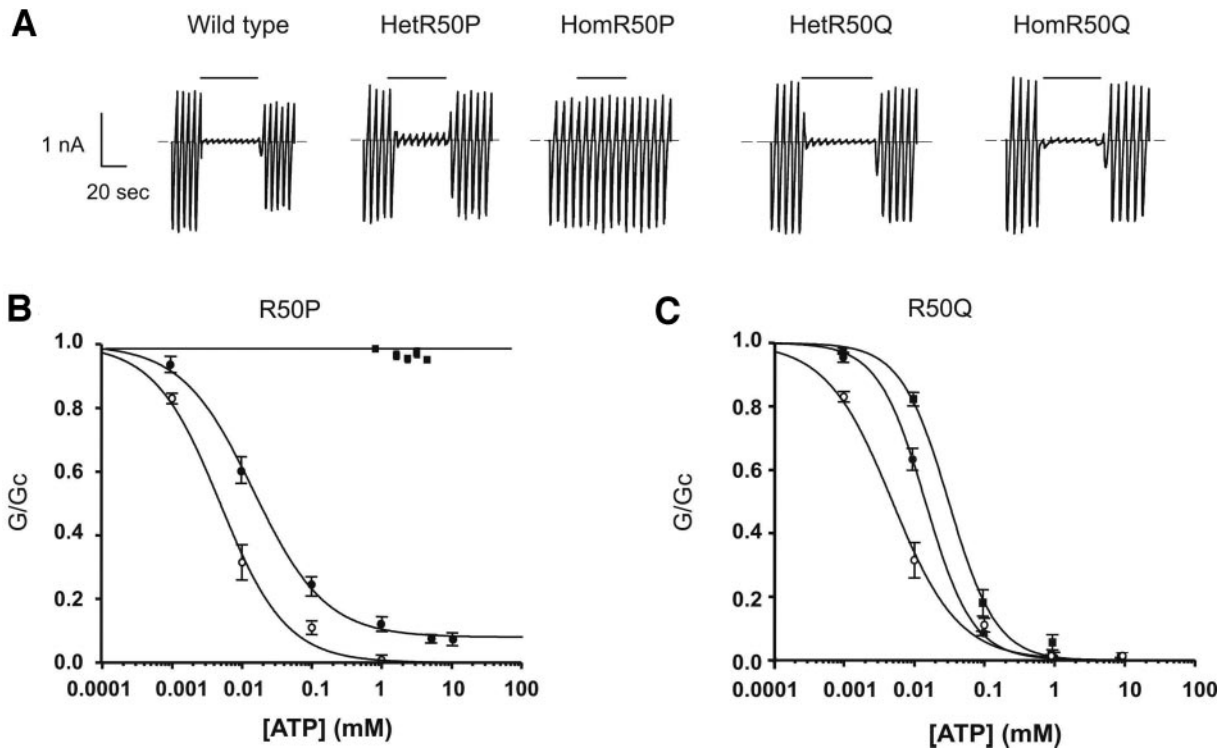

FIG. 3. ATP concentration-response relations in the absence of Mg^{2+}. *A*: K_{ATP} channel currents recorded in response to successive voltage ramps from −110 to +100 mV in an inside-out patch excised from a *Xenopus* oocyte coexpressing the indicated K_{ATP} channels. The dashed line indicates the zero current level. The bar indicates application of 1 mmol/l ATP. *B* and *C*: Mean relationship between [ATP] and K_{ATP} conductance (*G*), expressed relative to the conductance in the absence of nucleotide (G_c) for Kir6.2/SUR1 (○, $n = 7$) and heterozygous (●, $n = 8$) or homomeric (■, $n = 5$) Kir6.2-R50P/SUR1 channels (*B*) and for Kir6.2/SUR1 (○, $n = 7$), and heterozygous (●, $n = 6$) or homomeric (■, $n = 6$) Kir6.2-R50Q/SUR1 channels (*C*). The smooth curves are the best fit of Eq. 1 to the data, using the parameters given in Table 1 (Eq. 2; $a = 0.07$ for hetR50P channels). The line through the Kir6.2-R50P/SUR1 data was drawn by eye. All experiments were carried out in the absence of Mg^{2+}.

to this channel population as heterozygous channels. There was no significant difference between the amplitude of resting heterozygous R50Q (hetR50Q) and wild-type currents. In contrast, resting heterozygous R50P (hetR50P) currents were strikingly larger than wild type and not significantly different in amplitude from homR50P currents (Fig. 2). Tolbutamide blocked azide-activated currents by 84 ± 3% ($n = 11$) for hetR50P channels and 89 ± 4% ($n = 6$) for R50Q channels, compared with 95 ± 1% ($n = 6$) for wild-type channels.

Effects of R50 mutations on K_{ATP} channel ATP sensitivity. The increase in resting whole-cell K_{ATP} channel currents suggests that mutations at residue 50 may reduce the channel ATP sensitivity, as found for other PNDM mutations (1,2). Mutations at R50, including R50P and R50Q, have previously been shown to decrease ATP inhibition of homomeric channels coexpressed with (34,35) or without (36,37) SUR1. To compare the effect of these mutations on the homomeric channels studied previously with the heterozygous channels found in patients, we first measured ATP concentration-response curves in the absence of Mg^{2+}. This enables the effects of the mutation on ATP inhibition at Kir6.2 to be studied in isolation from effects mediated via SUR1.

Both mutations at R50 reduced the channel sensitivity to ATP, but they did so to different extents (Fig. 3). The ATP concentration-inhibition curves for both homR50Q and hetR50Q channels were well fit by the Hill equation (Eq. 1), with half-maximal block (IC_{50}) at 35 μmol/l for homR50Q and 16 μmol/l for hetR50Q, compared with 5 μmol/l for the wild-type channel (Table 1). However, homR50P channels were not blocked at all by ATP, even at concentrations as high as 10 mmol/l. The IC_{50} for ATP block of hetR50P channels was 17 μmol/l ATP, similar to that found for R50Q, but the block was not complete even at very high ATP concentrations. The hetR50P concentration-inhibition curve was best fit by assuming that in the heterozy-

TABLE 1
ATP-sensitivities of wild-type and mutant channels

	Mg^{2+} free		2 mmol/l Mg^{2+}	
Mutation	IC_{50} (mmol/l)	*h*	IC_{50} (mmol/l)	*h*
Kir6.2 (wild type)	5.4 ± 1 (7)	0.97 ± 0.12 (7)	28.3 ± 4.7 (6)	0.72 ± 0.05 (6)
Kir6.2-R50P (hetero)	16.9 ± 3.7 (8)*	0.88 ± 0.05 (8)	62.1 ± 6.1 (6)*	0.87 ± 0.11 (6)
Kir6.2-R50Q (hetero)	15.5 ± 1.5 (6)*	1.37 ± 0.04 (6)	128.3 ± 15.6 (9)*	0.77 ± 0.03 (9)
Kir6.2- R50Q (homo)	34.8 ± 6.6 (6)*	1.28 ± 0.12 (6)	784.6 ± 99.7 (6)*	1.40 ± 0.32 (6)

Data are means ± SE (*n*). ATP concentrations causing half-maximal block (IC_{50}) of wild-type heterozygous and homomeric Kir6.2/SUR1 mutant channels are measured in the absence and presence of Mg^{2+}. *$P < 0.01$ against wild-type channel. *h*, Hill coefficient.

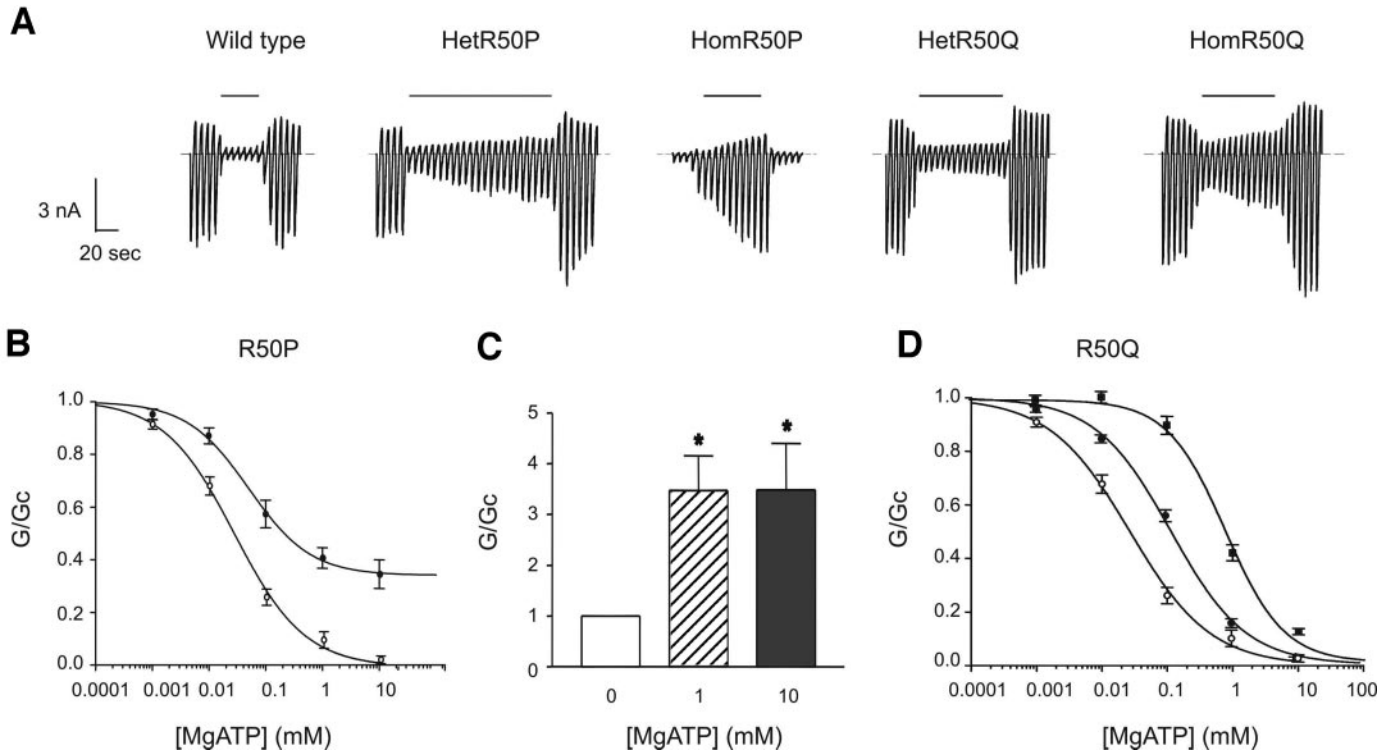

FIG. 4. ATP concentration-response relations in the presence of Mg^{2+}. *A*: K_{ATP} channel currents recorded in response to successive voltage ramps from −110 to +100 mV in an inside-out patch excised from a *Xenopus* oocyte expressing the indicated K_{ATP} channels. The dashed line indicates the zero current level. The bar indicates application of 1 mmol/l ATP. *B* and *D:* Mean relationship between [MgATP] and K_{ATP} channel conductance (*G*), expressed relative to the conductance in the absence of nucleotide (G_c) for Kir6.2/SUR1 (○, $n = 6$) and hetR50P (●, $n = 6$) channels (*B*) or for Kir6.2/SUR1 (○, $n = 6$), hetR50Q (●, $n = 9$), and homR50Q (■, $n = 5$) channels (*D*). The smooth curves represent the best fit of Eq. 1 to the data, using the parameters given in Table 1. For hetR50P data, Eq. 2 was used ($a = 0.28$). *C*: Effect of [MgATP] on homKir6.2-R50P/SUR1 channels ($n = 8$). Currents are expressed relative to their amplitude in the absence of nucleotide. *$P < 0.01$.

gous state, a small fraction of channels (7%) are never closed by ATP (Eq. 2; $a = 0.07$).

Effects on K_{ATP} channel ATP sensitivity in the presence of Mg^{2+}. Previous studies have shown that Kir6.2 mutations associated with neonatal diabetes may not only decrease the sensitivity of Kir6.2 to ATP but may also enhance channel activation by Mg^{2+} nucleotides (28,29). We therefore next explored the effect of R50 mutations on the ATP sensitivity of mutant channels in the presence of 2 mmol/l Mg^{2+} to more closely approximate the physiological condition (Fig. 4; Table 1).

The ATP sensitivity of R50Q channels was further reduced in the presence of Mg^{2+} (Fig. 4), the IC_{50} being ~5-fold greater for hetR50Q channels (128 μmol/l) and ~30-fold greater for homR50Q channels (875 μmol/l) than that of wild-type channels (28 μmol/l) (Table 1). Strikingly, homR50P currents were activated, rather than blocked, by ATP. This is not unexpected given that homR50P channels are not blocked in the absence of Mg^{2+} (Fig. 3) and that MgATP stimulates K_{ATP} channel activity via SUR1 (24). The IC_{50} for ATP inhibition of hetR50P channels (62 μmol/l) was actually less than that of hetR50Q channels (Table 1), but ~33% of the current was insensitive to ATP. This unblocked current was substantially larger than that found for hetR50P in the absence of Mg^{2+} (33 vs. 7%).

In both β-cells (rev. in 38) and oocytes (39), physiological MgATP concentrations range between 1 and 5 mmol/l. Table 2 shows that K_{ATP} channel currents are substantially greater at 1, 3, and 5 mmol/l MgATP for hetR50P channels than for wild-type channels. This can account for the larger resting currents observed in intact oocytes. The magnitude of the R50Q currents is smaller than that of R50P currents but still more than twice as large as for wild-type channels.

Mechanism of reduction in ATP sensitivity. Mutation of Kir6.2 residues can cause a reduction in ATP inhibition in several ways. It may disrupt ATP binding, impair the mechanism by which ATP binding is translated into channel closure, or stabilize the intrinsic (unliganded) open state of the channel. In the latter case, inhibition by ATP is

TABLE 2
K_{ATP} channel currents at physiological ATP concentrations

Mutation	1 mmol/l Mg-ATP	3 mmol/l Mg-ATP	5 mmol/l Mg-ATP
Kir6.2 (wild type)	7.5 ± 2.1 (6)	3.8 ± 1.4 (6)	2.7 ± 1.1 (6)
Kir6.2-R50P (hetero)	39.8 ± 4.8 (6)	36.5 ± 4.9 (6)	35.6 ± 4.8 (6)
Kir6.2-R50Q (hetero)	17.0 ± 1.4 (9)	8.2 ± 0.8 (9)	5.7 ± 0.6 (9)
Kir6.2-R50Q (homo)	43.0 ± 2.8 (6)	15.9 ± 2.8 (6)	9.8 ± 2.0 (6)

Data are % (*n*). Fraction of unblocked K_{ATP} conductance measured in excised patches in the presence of 1, 3, or 5 mmol/l MgATP for channels containing SUR1 and wild-type, heterozygous, and homomeric Kir6.2/SUR1 mutant channels.

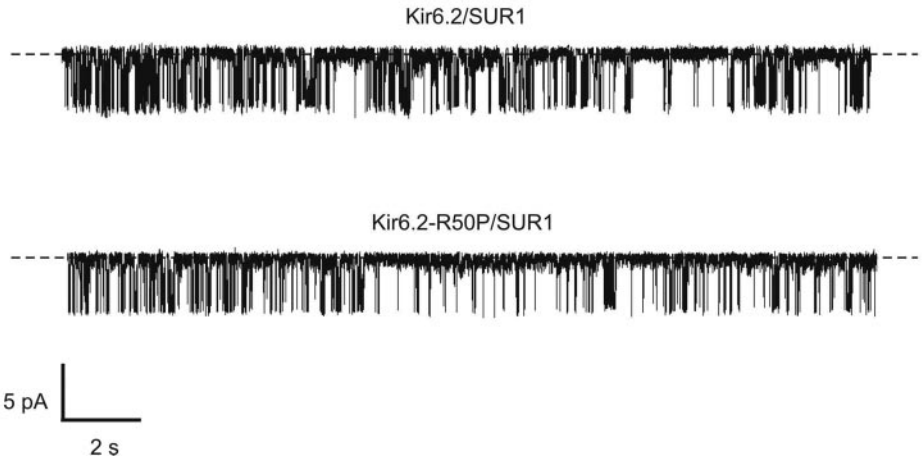

FIG. 5. Kinetics of wild-type and mutant K_{ATP} channels. Representative single–K_{ATP} channel currents recorded at −60 mV from inside-out patches from oocytes expressing SUR1 plus either Kir6.2 or Kir6.2-R50P.

affected indirectly: this is because ATP stabilizes the long-closed states of the channel, which occur less frequently in the mutant channel (30,31). Because R50 lies within the putative ATP-binding site (Fig. 1), mutations at this residue are likely to alter ATP binding. To determine whether they also alter the channel open probability, we examined the effect of the R50P mutation on single-channel currents in the absence of ATP, where intrinsic gating can be assessed. The intrinsic open probability of homR50P channels was not significantly different from wild-type channels (Fig. 5) (0.19 ± 0.06 [$n = 6$] for wild-type channels and 0.13 ± 0.01 [$n = 6$] for homR50P channels). This suggests that the R50P mutation primarily affects ATP binding and/or transduction.

DISCUSSION

Our results demonstrate that mutations at residue 50 of Kir6.2 cause neonatal diabetes by reducing the ability of MgATP to inhibit the K_{ATP} channel. The two different mutations at the same residue have different functional effects, and this is reflected in the severity of the clinical phenotype.

Functional implications. The large amplitude of hetR50P currents recorded in inside-out patches exposed to physiological levels of MgATP explains the dramatic increase in the whole-cell K_{ATP} channel currents observed in *Xenopus* oocytes before metabolic inhibition (resting currents). In pancreatic β-cells, a similar increase in the K_{ATP} channel current would be expected to cause membrane hyperpolarization and reduce or abolish the membrane depolarization evoked by glucose. This would prevent electrical activity, calcium influx, and insulin secretion and can thereby account for the diabetic phenotype of the patient. Although the increase in resting whole-cell K_{ATP} channel currents found for hetR50Q channels in oocytes is rather small, this may not necessarily be the case in β-cells, where resting ATP concentrations appear to be lower (38,39) and native wild-type K_{ATP} channels are open in the absence of glucose (19,40).

In many muscle and nerve tissues, K_{ATP} channels are normally shut and open only under metabolic stress (18). Thus, a greater reduction in ATP sensitivity is required to increase the resting K_{ATP} channel current sufficiently to influence electrical activity in these cells (1,2). Previous studies have shown that mutations that cause DEND syndrome give rise to heterozygous K_{ATP} channel currents that are much less sensitive to MgATP inhibition than mutations associated with neonatal diabetes alone (26–28). Thus in the presence of 3 mmol/l MgATP, between 34 and 50% of the heterozygous current is not blocked, compared with <1% for wild-type channels and 6 to 15% for PNDM mutant channels (28). The R50P mutation results in MgATP-insensitive currents of a similar magnitude (37%) to those that cause DEND syndrome. The clinical features of the patient with the R50P mutation include all components of the DEND syndrome: developmental delay, epilepsy, and neonatal diabetes. However the neurological features might also be sequelae of probable cerebral edema at the time of initial treatment. It is not possible to resolve the relative roles of these two etiologies. The marked delay in the onset of walking is typically seen in DEND syndrome and thus entirely consistent with the greater functional severity of the Kir6.2-R50P mutation. Likewise, the clear lack of developmental or neurological problems found in patient 2 (R50Q) is harmonious with the smaller decrease in K_{ATP} channel ATP sensitivity produced by this mutation (8% unblocked current in 3 mmol/l MgATP). Other features consistent with R50P resulting in a more severe β-cell phenotype than R50Q are the lower birth weight, earlier age of diagnosis, and more severe hyperglycemia.

Molecular mechanism of reduced ATP sensitivity. Unlike DEND syndrome mutations analyzed to date, the R50P mutation did not alter the single-channel kinetics (Fig. 5). Other mutations at R50 also do not alter the kinetics of Kir6.2ΔC (a truncated version of Kir6.2) when expressed in the absence of SUR1 (36). This suggests that mutations at R50 affect either ATP binding and/or the mechanism by which ATP binding is translated into closing of the Kir6.2 pore. This idea is consistent with the proposed location of R50 within the ATP-binding site (37,41) and with functional studies that suggest that R50 interacts with the γ-phosphate of ATP (34–37).

In a molecular model of Kir6.2 (41), the ATP-binding pocket lies at the interface between the NH_2- and COOH-terminal domains of adjacent subunits, and the positively charged side-chain of R50 interacts electrostatically with the negatively charged γ-phosphate of ATP (Fig. 1). This interaction is supported by functional studies (34–37). The position of R50 in the model is such that this residue must move if ATP is to enter its binding site (which is formed principally by the adjacent subunit). Thus R50 appears to act as a "gate," allowing access of ATP into its site and locking the molecule in once it has bound. It is important to note that R50 does not occlude the end of the binding pocket, which explains why large moieties can be added to

the terminal phosphate of ATP without compromising the channel ATP sensitivity (42). Rather, the residue acts in the manner of a cleat that prevents a rope from moving.

Mutation of R50 to Q reduces the IC_{50} for ATP inhibition of homomeric K_{ATP} channels approximately sevenfold, whereas the R50P mutation abolished ATP block completely. It is likely that neutralization of R50 (to glutamine) prevents electrostatic interaction with ATP without markedly affecting the protein structure. However, proline is known to cause marked conformational changes in proteins, often being associated with turns or loops, which may be why it disrupts ATP binding so dramatically. Interestingly, R50P is the first mutation to be shown to totally prevent ATP block in the absence of Mg^{2+}.

Origin of the ATP-insensitive current through hetR50P channels. In the heterozygous state, R50P channels are not completely blocked by ATP, even at saturating concentrations of the nucleotide (Figs. 3 and 4). The heterozygous channel population is composed of homomeric wild-type channels, heteromeric channels containing both wild-type and mutant subunits, and homomeric mutant channels. If wild-type and mutant subunits associate randomly, their distribution should obey the binomial equation and 1/16 of channels will contain only mutant subunits. Because binding of ATP to a single subunit within the tetramer is sufficient to close the channel (43), only channels containing all mutant subunits will show a reduced ATP sensitivity. HomR50P channels are totally insensitive to ATP. Assuming that wild-type and mutant subunits express at similar levels, binomial analysis predicts that in the absence of Mg^{2+}, 6.3% of the current will be insensitive to ATP. This is very close to the measured value of 7%.

A much greater fraction of unblocked current was observed for hetR50P channels in the presence of Mg^{2+} (Fig. 4*B*). This reflects the fact that MgATP has both inhibitory and stimulatory effects on K_{ATP} channels and that the stimulatory effect is unmasked when ATP inhibition at Kir6.2 is abolished, as occurs with the R50P mutation. Given that mutant homomeric mutant channels make up ~7% of the total population and that hom50P channels are activated 3.5-fold by 10 mmol/l MgATP (Fig. 4*C*), the fraction of unblocked current must be at least 25%. The larger current reflects the fact that MgATP also activates wild-type and heteromeric channels, thus further increasing the currents at high MgATP concentrations.

Tolbutamide block. Both hetR50P and homR50P currents showed reduced tolbutamide block. This may indicate impaired coupling of SUR1 to Kir6.2. However, it may also be a secondary consequence of the mechanism of tolbutamide block. In the absence of nucleotides, the maximal K_{ATP} channel block produced by sulfonylureas is only ~60%, but this is increased (to ~100%) in the presence of Mg^{2+} nucleotides (33). This is because sulfonylureas reduce the stimulatory effect of Mg^{2+} nucleotides, thereby unmasking their inhibitory effect at the ATP-binding site on Kir6.2. This adds to the block produced by the sulfonylurea, causing an apparent increase in block. Because the R50P mutation substantially reduces the inhibitory effect of ATP, this increase will be less, and consequently, tolbutamide block in the intact cell (where Mg^{2+} nucleotides are present) will be less for the mutant channel.

Clinical implications. Sulfonylureas are an effective therapy for most patients with *KCNJ11* mutations that cause neonatal diabetes alone, and many have now been successfully transferred to sulfonylurea therapy from insulin (3,5,9–11,44). In functional studies, K_{ATP} channels carrying these mutations remain as sensitive to tolbutamide inhibition as wild-type channels, being inhibited between 89 and 96% by 0.5 mmol/l tolbutamide (4,26,29). Mutations that cause DEND syndrome are far less sensitive to sulfonylureas: for example, tolbutamide only blocks V59G channels by 40% (27). This is because all DEND mutations analyzed to date act by increasing the channel's intrinsic open probability, which has the secondary consequence that both ATP and sulfonylureas are less effective as blockers (30,31). In contrast, the R50P mutation does not alter the single-channel kinetics, which explains why the channel remains sensitive to sulfonylureas, with 0.5 mmol/l tolbutamide causing ~85% block. This is presumably why sulfonylureas were able to treat the patient's diabetes. Importantly, assuming that it is able to access target tissues, the drug may be able to ameliorate the muscle, neurological, and developmental symptoms as well as the diabetes. The observations that not all Kir6.2 mutations associated with developmental delay influence channel gating and that some may be sensitive to tolbutamide block suggest that it is worth trying sulfonylureas even in the most severe cases when novel mutations are detected.

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