Original Article

A Kir6.2 Mutation Causing Neonatal Diabetes Impairs Electrical Activity and Insulin Secretion From INS-1 β-Cells

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ATP-sensitive K^+ channels (K_{ATP} channels) couple β-cell metabolism to electrical activity and thereby play an essential role in the control of insulin secretion. Gain-of-function mutations in Kir6.2 (*KCNJ11*), the pore-forming subunit of this channel, cause neonatal diabetes. We investigated the effect of the most common neonatal diabetes mutation (R201H) on β-cell electrical activity and insulin secretion by stable transfection in the INS-1 cell line. Expression was regulated by placing the gene under the control of a tetracycline promoter. Transfection with wild-type Kir6.2 had no effect on the ATP sensitivity of the K_{ATP} channel, whole-cell K_{ATP} current magnitude, or insulin secretion. However, induction of Kir6.2-R201H expression strongly reduced K_{ATP} channel ATP sensitivity (the half-maximal inhibitory concentration increased from ~20 μmol/l to ~2 mmol/l), and the metabolic substrate methyl succinate failed to close K_{ATP} channels or stimulate electrical activity and insulin secretion. Thus, these results directly demonstrate that Kir6.2 mutations prevent electrical activity and insulin release from INS-1 cells by increasing the K_{ATP} current and hyperpolarizing the β-cell membrane. This is consistent with the ability of the R201H mutation to cause neonatal diabetes in patients. The relationship between K_{ATP} current and the membrane potential reveals that very small changes in current amplitude are sufficient to prevent hormone secretion. *Diabetes* 55: 3075–3082, 2006

Neonatal diabetes is a rare inherited form of diabetes that manifests within the first 6 months of life (1,2). Approximately 50% of cases of neonatal diabetes 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). Functional studies of these mutations in heterologous systems have shown that they result in a reduction in the ability of ATP to close the K_{ATP} channel (10–16).

In pancreatic β-cells, a proportion of K_{ATP} channels is open at substimulatory glucose concentrations (17). The resulting K^+ efflux holds the β-cell membrane at a hyperpolarized potential, preventing electrical activity and insulin secretion (18). Elevation of the plasma glucose concentration stimulates glucose uptake and metabolism by the β-cell, causing an increase in ATP and an accompanying decrease in MgADP. These changes in adenine nucleotide concentrations close K_{ATP} channels and, as a consequence, elicit membrane depolarization, opening of voltage-gated Ca^{2+} channels, and Ca^{2+}-dependent electrical activity. This leads to increased Ca^{2+} influx and insulin release (18). Mutations that render the K_{ATP} channel less sensitive to inhibition by ATP are therefore predicted to prevent β-cell depolarization, electrical activity, and insulin secretion in response to increased oxidative metabolism. The observation that a glucose challenge fails to elicit a rise in plasma C-peptide or insulin in patients carrying neonatal diabetes mutations is consistent with this idea (3,5,9,10). However, there is no direct evidence for the hypothesis.

To date, most functional analyses of *KCNJ11* mutations causing neonatal diabetes have been carried out by heterologous expression of wild-type or mutant K_{ATP} channels in *Xenopus* oocytes (10–16). Although the ATP sensitivity of the wild-type channel does not appear to be affected by the expression system (10–16,19), it is not possible to extrapolate the relationship between β-cell metabolism and K_{ATP} channel activity from oocyte studies. Here, we used INS-1 cells, an insulin-secreting β-cell line, to directly show that the R201H mutation in Kir6.2, which is a common cause of neonatal diabetes, leads to K_{ATP} channels with reduced ATP sensitivity and that this, in turn, prevents membrane depolarization, electrical activity, and insulin secretion in response to metabolic substrate.

RESEARCH DESIGN AND METHODS

Pancreatic β-cell (INS-1) lines that conditionally overexpress wild-type Kir6.2 or Kir6.2-R201H were constructed using the Flp-In T-REx system (Invitrogen) developed by Thomas et al. (20). cDNAs encoding wild-type or mutant Kir6.2 were initially cloned downstream of the Tet operator into the plasmid pcDNA5/FRT/TO (Invitrogen), using the Pme1 restriction site. Plasmids were sequenced to confirm the correct orientation of the ligated cDNA and then transfected into INS-1 Flp-In T-REx cells using TransFAST reagent. Cells were cotransfected with pOG44 plasmid encoding Flp recombinase (Invitrogen) to facilitate integration into the Flp recombination site. This method has the advantage that both the wild-type and mutant cDNAs are integrated at the same site within the genomic DNA, which means that clonal selection is not

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K_{ATP} channel, ATP-sensitive K^+ channel.

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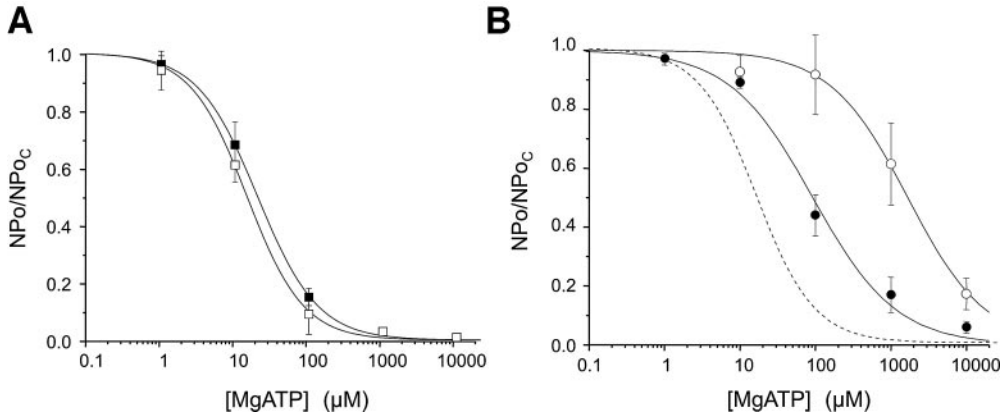

FIG. 1. *A*: Mean ATP concentration-inhibition relationships for K_{ATP} currents in INS-1 cells stably transfected with wild-type Kir6.2 before (■, $n = 7$) and after (□, $n = 7$) induction of expression with 50 ng/ml tetracycline. The curves are the best fit of the Hill equation to the mean data with $IC_{50} = 20$ μmol/l, $h = 1.1$ (■), and with $IC_{50} = 15$ μmol/l, $h = 1.3$ (□). *B*: Mean ATP concentration-inhibition relationships for K_{ATP} currents in INS-1 cells stably transfected with Kir6.2-R201H before (●, $n = 6$) and after (○, $n = 4$) induction with tetracycline. The curves are the best fit of the Hill equation to the mean data with $IC_{50} = 92$ μmol/l, $h = 0.79$ (●), and with $IC_{50} = 1.7$ mmol/l, $h = 0.84$ (○). The dashed line indicates the concentration-inhibition curves for wild-type channels shown in *A*.

required because all stably transfected cells are functionally equivalent. Stable cell lines expressing the cDNAs were obtained by hygromycin (150 μg/ml) selection and were expanded in tissue culture. Induction of expression of Kir6.2 was confirmed by Western blotting with anti-Kir6.2 serum (a kind gift from Dr. R.I. Norman, University of Leicester) after addition of tetracycline.

Cell culture. INS-1 cells were cultured in RMPI 1640 medium containing 11 mmol/l glucose supplemented with 5 mmol/l glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 10% FCS, 50 μmol/l β-mercaptoethanol, and 150 μg/ml hygromycin (to maintain the selection) at 37°C in a fully humidified atmosphere of 5% CO_2. To induce expression, cells were treated with tetracycline (50 ng/ml) for 24 h.

Electrophysiology. Currents were recorded using an EPC7 patch-clamp amplifier (List Elektronik, Darmstadt, Germany). Single-channel currents were recorded from inside-out membrane patches at −60 mV, filtered at 1 kHz, digitized at 2 kHz, and analyzed using Clampfit-9.2 software (Axon Instruments, Union City, CA). Channel activity was expressed as NP_O, where N is the number of channels in the patch and P_O the open probability. Whole-cell currents were recorded at a holding potential of −70 mV in response to hyperpolarizing 20-mV voltage steps. Currents and conductances were normalized to cell capacitance to correct for differences in cell size. There was no significant difference in cell capacitance between the four groups of cells, which was (in pF): 7.0 ± 0.5 (wild type), 6.9 ± 0.4 (wild type plus tetracycline), 6.3 ± 0.6 (R201H), and 7.2 ± 0.8 (R201H plus tetracycline).

For inside-out patch recordings, the pipette solution contained (in mmol/l): 140 KCl, 10 HEPES (pH 7.2 with KOH), 1.1 $MgCl_2$, and 2.6 $CaCl_2$. The intracellular (bath) solution contained (in mmol/l): 107 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 11 EGTA, and 10 HEPES (pH 7.2 with KOH), plus MgATP as indicated. The pH was measured after ATP addition and readjusted if required. For whole-cell experiments (perforated patch and standard whole cell), the pipette solution contained (in mmol/l): 76 K_2SO_4, 10 NaCl, 10 KCl, 1 $MgCl_2$, and 5 HEPES (pH 7.35 with KOH), plus 0.24 mg/ml amphotericin. No ATP was added. The bath solution contained (in mmol/l): 137 NaCl, 5.6 KCl, 10 HEPES (pH 7.4 with NaOH), 2.6 $CaCl_2$, and 1.1 $MgCl_2$. Recordings were initiated 30 min after exposure to substrate-free solutions at 37°C. The duration of exposure to each substrate concentration was >2 min. All experiments were conducted at 21–23°C, and the bath solution was perfused continuously.

ATP concentration-inhibition curves were fit with the Hill equation: $NP_O/NP_{OC} = [1 + ([ATP]/IC_{50})^h]^{-1}$, where IC_{50} is the half-maximal inhibitory concentration of ATP, h is the Hill coefficient, and NP_{OC} is the channel activity in nucleotide-free solution.

For Fig. 5, the membrane potential was first measured in current clamp mode. The whole-cell conductance was then measured (between −90 and −50 mV) by switching to voltage clamp.

Insulin secretion. INS1 cells expressing wild-type or R201H Kir6.2 were plated at a density of 10^5 cells per well in 24-well plates, cultured for 24 h before induction with tetracycline (0 or 50 ng/ml), and assayed 24 h after induction. Cells were incubated for 60 min in Krebs-Ringer buffer (in mmol/l: 118.5 NaCl, 2.54 $CaCl_2$, 1.19 KH_2PO_4, 4.74 KCl, 25 $NaHCO_3$, 1.19 $MgSO_4$, and 10 HEPES) with 0, 1, 5, and 20 mmol/l methyl succinate or 0.5 mmol/l tolbutamide. We performed six to eight replicates for each test solution. The supernatant was assayed for insulin using a mouse insulin enzyme-linked immunosorbent assay kit (Mercodia). Insulin content was determined by overnight extraction of cells using cold 19:1 ethanol/acetic acid. Secretion was normalized to insulin content to correct for any difference in the number of cells.

Data are the means ± SE. The IC_{50} (and h) values given in Table 1 and RESULTS were obtained by fitting each concentration-inhibition curve individually and then taking the mean of the parameter values obtained. The smooth curves shown in Fig. 1 were obtained by fitting the mean data. The Mann-Whitney U test was used to determine statistical significance; values of $P < 0.05$ were considered significant.

RESULTS

ATP sensitivity of wild-type and mutant K_{ATP} channels. Figure 1*A* shows ATP concentration-inhibition curves measured for INS-1 cells transfected with wild-type Kir6.2 under the control of a tetracycline promoter. Before induction of Kir6.2 by tetracycline, the IC_{50} for ATP block was 25 μmol/l (Table 1), which is within the range of that found previously for Kir6.2/SUR1 channels heterologously expressed in *Xenopus* oocytes (10–35 μmol/l) (4,21). The IC_{50} was unchanged (17 μmol/l) after induction with tetracycline (Fig. 1, Table 1).

When cells were transfected with Kir6.2-R201H, there was a slight but significant reduction in K_{ATP} channel ATP sensitivity even before the addition of tetracycline. The IC_{50} increased from 25 to 54 μmol/l (Fig. 1*B*, Table 1). This suggests that the tetracycline promoter is slightly "leaky" and that a small fraction of mutant channel subunits are expressed. However, it is likely that the expression of Kir6.2-R201H is somewhat less than that of native Kir6.2 because coexpression of a 1:1 mix of wild-type and R201H Kir6.2 mRNAs in *Xenopus* oocytes caused a larger increase in the IC_{50} (to 143 μmol/l) (4).

Induction of expression of Kir6.2-R201H with 50 ng/ml

TABLE 1
Inhibitory effects of MgATP

Kir6.2 genotype	Tetracycline (ng/ml)	IC_{50} (μmol/l)	h	n
WT	0	24.9 ± 6.1	1.2 ± 0.2	7
WT	50	17.2 ± 7.6	1.6 ± 0.3	7
R201H	0	54.2 ± 7.3*	1.3 ± 0.2	6
R201H	50	2,021 ± 852*	3.1 ± 2.2	4

Data are means ± SE, unless otherwise indicated. *$P < 0.05$ against wild-type (WT) equivalent. h, Hill coefficient; n, number of cells.

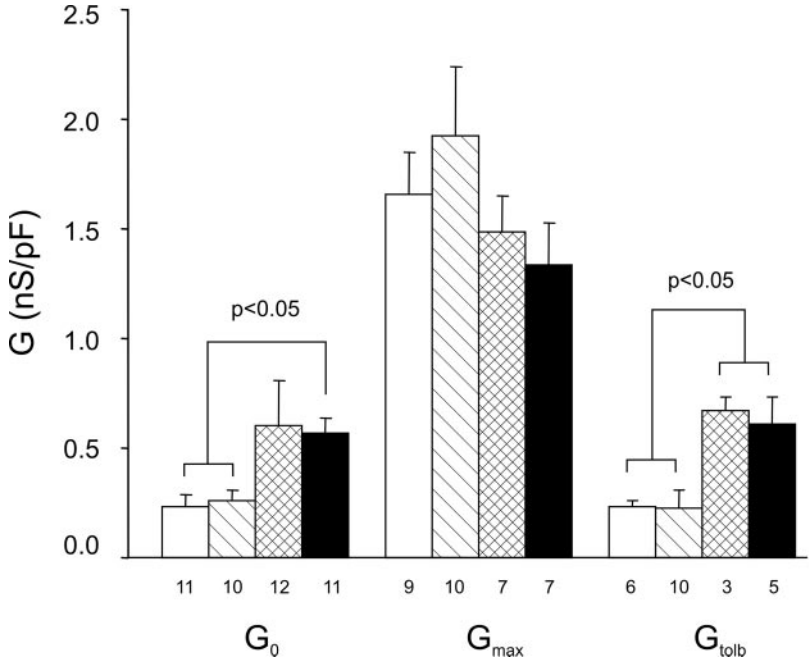

FIG. 2. Mean K_{ATP} conductances recorded from INS-1 cells expressing wild-type Kir6.2 in the absence (□) or presence (▨) of tetracycline, or expressing Kir6.2-R201H in the absence (▨) or presence (■) of tetracycline. The number of cells is given below the columns. G, conductance; G_0, resting conductance, recorded in the perforated-patch configuration in glucose-free external solution; G_{max}, maximal K_{ATP} conductance, recorded in the standard whole-cell configuration after ATP washout; G_{tolb}, K_{ATP} conductance, recorded in the standard whole-cell configuration after ATP washout, in the presence of 0.5 mmol/l tolbutamide.

tetracycline reduced the ATP sensitivity further and shifted the IC_{50} to 2.0 ± 0.9 mmol/l (Fig. 1*B*, Table 1). This value is not different from that found for homomeric Kir6.2-R201H/SUR1 channels expressed in *Xenopus* oocytes (2.0 mmol/l) (4), which suggests that under these conditions, the exogenously expressed Kir6.2-R201H outcompetes the native wild-type Kir6.2, so that the resulting channels are effectively composed entirely of mutant subunits.

In the rest of this article, we refer to cells transfected with Kir6.2-R201H, but not induced by tetracycline, as R201H cells, and we refer to cells induced with tetracycline as tetR201H cells. Cells transfected with wild-type Kir6.2 that were or were not exposed to tetracycline are referred to as WT and tetWT cells, respectively. Because tetracycline did not affect K_{ATP} channel ATP sensitivity, we confined our electrophysiological studies of wild-type Kir6.2 to WT cells (i.e., no tetracycline).

Whole-cell K_{ATP} currents are larger for cells expressing mutant channels. We next measured the magnitude of whole-cell K_{ATP} currents in INS-1 cells using the perforated-patch configuration of the patch-clamp method, which retains cell metabolism intact (22,23). After a 30-min preincubation in substrate-free solution, R201H and tetR201H cells had resting conductances that were about twofold greater than cells expressing wild-type Kir6.2 (Fig. 2). This is consistent with the lower ATP sensitivity of Kir6.2-R201H channels.

Following the perforated-patch recordings, the whole-cell configuration was established, and the maximal amplitude of the K_{ATP} current was measured. This was achieved by allowing ATP to wash out from the inside of the cell and was signaled by a marked increase in current amplitude. There was no significant difference between the maximal washout conductances recorded from WT, tetWT, R201H, or tetR201H cells (Fig. 2). This suggests that the K_{ATP} conductance magnitude is limited by the level of endogenous expression of the regulatory subunit of the K_{ATP} channel, SUR1: previous studies have shown that Kir6.2 does not traffic to the membrane in the absence of SUR1 (24). In the case of tetR201H cells, the resting conductance measured in glucose-free solution was 40% of the maximal K_{ATP} current. The resting R201H conductance was also 40% of maximal. This compares favorably to ~30% measured for homomeric R210H channels in *Xenopus* oocytes (4), particularly when one considers that resting ATP concentrations are higher in oocytes than mammalian cells (25,26). In contrast, WT and tetWT currents were only ~15% of maximal.

Whole-cell currents were blocked by 500 μmol/l tolbutamide, confirming they flow through K_{ATP} channels. However, R201H and tetR201H channels were less sensitive to tolbutamide, being blocked by only 45% compared with 86% for wild-type channels (Fig. 2).

Metabolic inhibition of whole-cell K_{ATP} currents. We next measured the concentration-inhibition curve for metabolic inhibition of wild-type and mutant K_{ATP} channels, again using the perforated-patch configuration to preserve β-cell metabolism. Because the INS-1 Flp-In T-REx clone 1–1.2 retains only a poor insulin secretory response to glucose but remains responsive to methyl succinate (27), we used the latter as a metabolic substrate. Unlike some other mitochondrial substrates (28), methyl succinate (20 mmol/l) did not affect K_{ATP} channel activity either when applied directly to the inside of an excised inside-out patch (Fig. 3*A*) or when added to the external solution in standard whole-cell recordings (Fig. 3*B*). Thus, we deduce that any effect of methyl succinate on perforated-patch whole-cell K_{ATP} currents must be a consequence of substrate metabolism.

In the absence of substrate, K_{ATP} conductances were significantly smaller for WT cells than for either R201H or

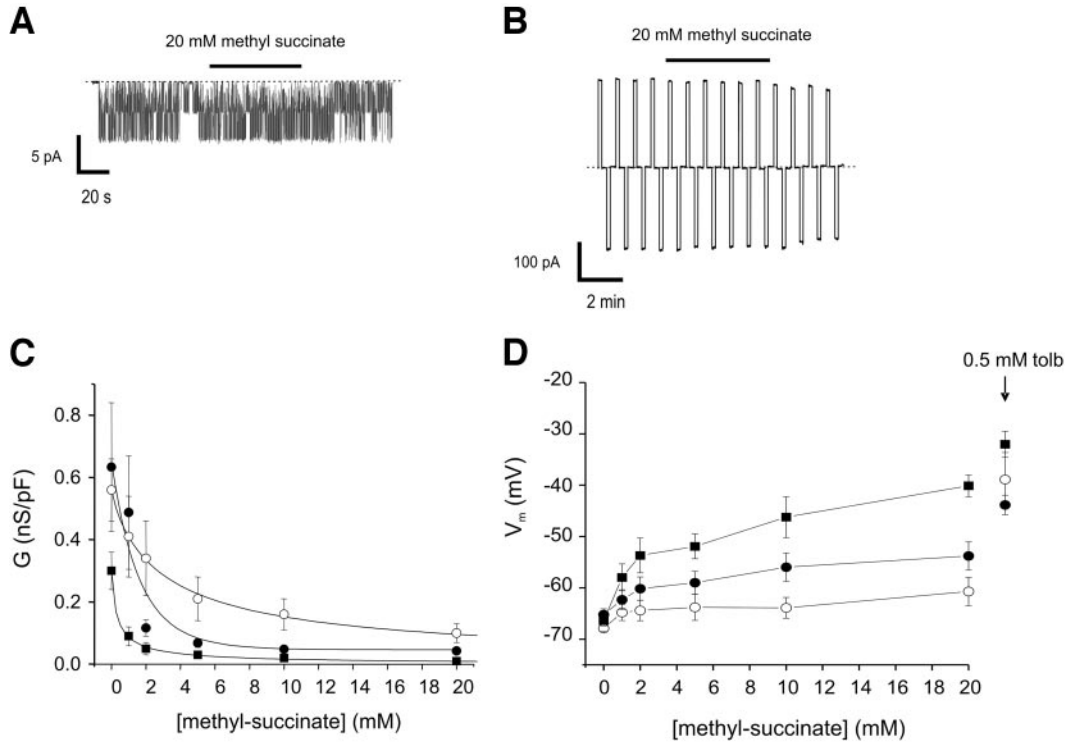

FIG. 3. *A*: Single–K_{ATP} channel currents recorded from an inside-out patch at −60 mV from INS-1 cells transfected with wild-type Kir6.2. Methyl succinate (20 mmol/l) was added as indicated. *B*: Whole-cell currents recorded in the standard whole-cell configuration from INS-1 cells transfected with wild-type Kir6.2. Methyl succinate (20 mmol/l) was added as indicated. *C* and *D*: Dependence of K_{ATP} conductance (*C*) and membrane potential (*D*) on the methyl succinate concentration, recorded from INS-1 cells transfected with wild-type Kir6.2 (■, $n = 7$) or Kir6.2-R201H before (●, $n = 11$) and after (○, $n = 7$) induction of Kir6.2-R201H with 50 ng/ml tetracycline. The membrane potential in the presence of 0.5 mmol/l tolbutamide is indicated. G, conductance; V_m, membrane potential.

tetR201H cells (Fig. 3*C*), as expected, because they are more ATP sensitive. The ability of metabolism to inhibit the whole-cell K_{ATP} conductance was also impaired when cells were transfected with Kir6.2-R201H (Fig. 3*C*). The IC_{50} for K_{ATP} current block was 0.7 ± 0.3 mmol/l ($n = 7$) for WT cells, 1.3 ± 0.3 mmol/l ($n = 12$) for R201H cells, and 3.5 ± 1.2 mmol/l ($n = 7$) for tetR201H cells. These differences were inversely correlated with the extent of membrane depolarization produced by methyl succinate.

As shown in Fig. 3*D*, there was no difference in the resting membrane potential of WT, R201H, or tetR201H cells, which was −66 ± 1 ($n = 7$), −68 ± 1 ($n = 7$), and −66 ± 2 ($n = 7$) mV, respectively. In WT cells, methyl succinate produced a marked membrane depolarization, and concentrations ≥5 mmol/l elicited action potential firing, with a threshold of around −50 mV (Figs. 3*D* and 4*A*). In contrast, in R201H and tetR201H cells, the membrane potential did not reach the threshold for action potential firing, even at the highest concentration of methyl succinate (Fig. 4*A* and *B*). However, tolbutamide depolarized cells expressing all three channel types, and it initiated action potentials.

It is noteworthy that at methyl succinate concentrations ≥10 mmol/l, the difference between the magnitude of the K_{ATP} conductance for WT and R201H channels was very small and did not reach significance. However, there was a large and significant difference in membrane potential (−46 and −58 mV, respectively). This is a function of the relationship between the K_{ATP} conductance and membrane potential. As shown in Fig. 5, this was highly nonlinear, so that when the K_{ATP} conductance was <0.5 nS (or ~0.1 nS/pF), very small changes in membrane potential produced a marked depolarization. Electrical activity was observed when the K_{ATP} conductance fell below 0.32 ± 0.03 nS ($n = 14$); the maximal conductance at which electrical activity was observed was 0.57 nS.

Insulin secretion measurements. Figure 6 illustrates the effect of expression of mutant Kir6.2 on insulin secretion in response to methyl succinate. Secretion was normalized to insulin content. Interestingly, the mean insulin content was somewhat lower in cells transfected with Kir6.2-R201H than in cells transfected with wild-type Kir6.2. However, it was not altered by tetracycline. The mean insulin content (μg/10^6 cells) in glucose-free Krebs-Ringer was 6.0 ± 0.3 (WT), 6.7 ± 0.7 (tetWT), 3.6 ± 0.4 (R201H), and 4.5 ± 1.0 (tetR201H). There was no significant difference in insulin content for cells of the same type incubated in different concentrations of secretagogue.

In the absence of substrate, basal insulin secretion was similar for all types of cells, although there was a slight but significant increase in R201H cells. Increasing methyl succinate to 5 or 20 mmol/l caused a significant increase in insulin secretion from WT or tetWT, but it had no significant effect on R201H or tetR201H cells. This was expected because methyl succinate fails to stimulate electrical activity in these cells (Fig. 4*B*). Tolbutamide (added in the presence of 5 mmol/l methyl succinate) had no additional effect on insulin secretion in WT or tetWT, but it significantly stimulated insulin secretion in tetR201H cells.

DISCUSSION

Our results show that gain-of-function mutations in Kir6.2 lead to a reduced sensitivity of the K_{ATP} channel to

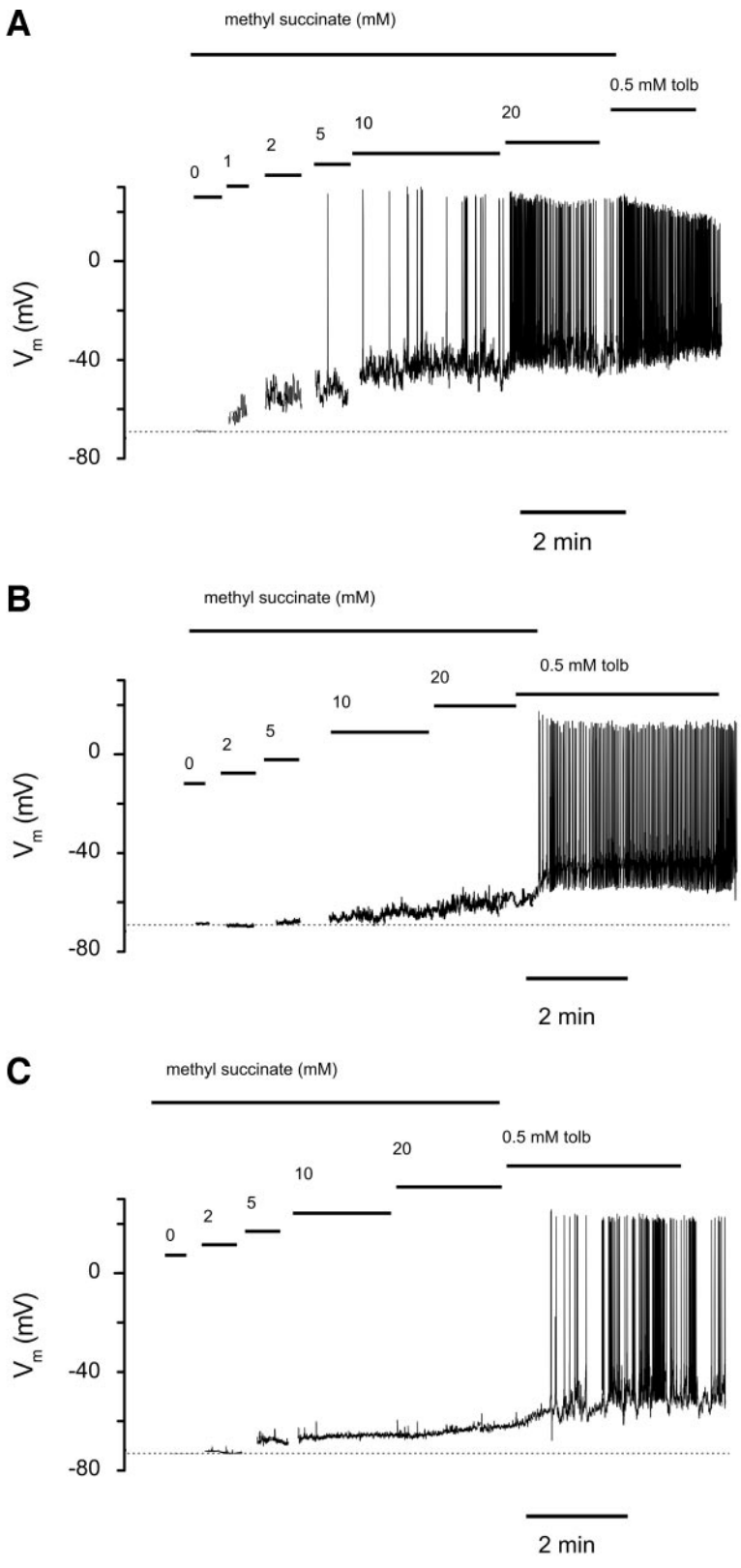

FIG. 4. Representative membrane potential (V_m) recordings from INS-1 cells expressing wild-type (*A*) or R201H (*B*) or tetR201H (*C*) channels. Methyl succinate and tolbutamide (tolb) were added as indicated.

inhibition by ATP when expressed in mammalian cells, as they do in *Xenopus* oocytes (10–16). Furthermore, they provide the first direct demonstration that this loss of ATP sensitivity leads to a reduced membrane depolarization in β-cells and to a loss of electrical activity and insulin secretion in response to a metabolic stimulus.

Resting K_{ATP} channel currents. Previously, measurements of ATP sensitivity of mutant Kir6.2 subunits have

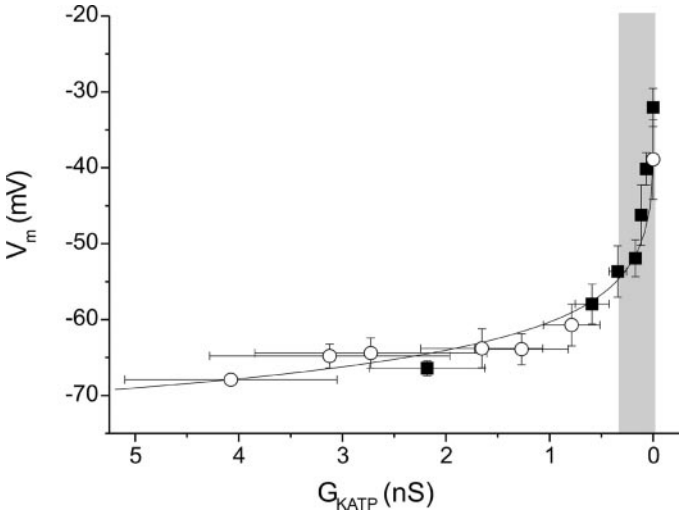

FIG. 5. Relationship between membrane potential (V_m) and K_{ATP} conductance (G_{KATP}) for wild-type Kir6.2 (■, n = 7) and tetR201H (○, n = 7). The gray shading indicates K_{ATP} conductance levels associated with electrical activity.

been largely confined to *Xenopus* oocytes. Our current data indicate that the oocyte provides a good substitute for the β-cell when measuring this parameter in excised patches. After induction with tetracycline, the IC_{50} values obtained in β-cells (Table 1) were similar to those found for oocytes expressing homomeric WT or R210H channels (4). Previous studies have shown that R201H subunits do not exert a dominant-negative effect on wild-type subunits (indeed, heterozygous channels more closely approximate the wild-type phenotype) (1–4). Thus, it appears that heterologously expressed Kir6.2 subunits dominate the population and that tetWT and tetR201H cells primarily express homomeric channels.

There is, however, a marked difference in the resting whole-cell currents measured in intact oocytes (by two-electrode voltage clamp) and INS-1 cells (perforated patch). In the latter, ~15% of the total K_{ATP} current (measured after washout of ATP in standard whole-cell recordings) was present in glucose-free solutions for WT and tetWT channels, and >40% was present for R201H and tetR201H channels. By contrast, when wild-type K_{ATP} currents are expressed in oocytes, the resting currents are too small to measure accurately (4). The amplitude of the maximal K_{ATP} current in oocytes is not easy to estimate. Currents recorded in the presence of azide may underestimate the total K_{ATP} current because azide has an inhibitory effect on the channel (29,30) and does not reduce the ATP concentration to zero (26,29). However, if we take the azide-activated current as a lower estimate of the maximal current, R201H currents are 40% activated at rest. Given the caveats stated above, it therefore appears that the resting currents in INS-1 cells are larger than in oocytes, perhaps because intracellular ATP concentrations are lower. Estimates of ATP concentrations in oocytes exposed to glucose-free solution are ~3–5 mmol/l (25,29) compared with ≤1 mmol/l in β-cells (26).

It is of interest that overexpression of wild-type Kir6.2 (tetWT) did not result in an increase in the whole-cell K_{ATP} current, probably because surface expression of Kir6.2 is limited by the level of SUR1 expression (24). This finding is of importance because it suggests that mutations that simply enhance expression of Kir6.2 alone (or indeed SUR1 alone) will not lead to changes in the magnitude of the K_{ATP} current or insulin secretion.

Implications for heterozygous patients. Transfection with Kir6.2-R201H reduced the ATP sensitivity of the K_{ATP} channel even when tetracycline was not used to induce expression. This indicates that the promoter is slightly leaky, a situation that has proven highly advantageous for our purposes because it provides an indication of what happens when only a small fraction of K_{ATP} channel subunits in the population carry the R201H mutation. All Kir6.2 neonatal diabetes mutations studied to date have been heterozygous, so that patients will express both wild-type and mutant subunits in their β-cells. In oocytes, coinjection of a 1:1 mixture of Kir6.2 and Kir6.2-R201H (with SUR1), to simulate the heterozygous state, produced K_{ATP} channels with an IC_{50} of 130 μmol/l for ATP inhibition (4). This is somewhat greater than the IC_{50} we observed for R201H cells (55 μmol/l), indicating that we probably do not achieve equal numbers of wild-type and mutant subunits in the noninduced state. Nevertheless, R201H cells did not respond to metabolic substrates with either electrical activity or insulin secretion. This was because even high concentrations of methyl succinate induced only a small membrane depolarization, which did not reach threshold for electrical activity. The data are

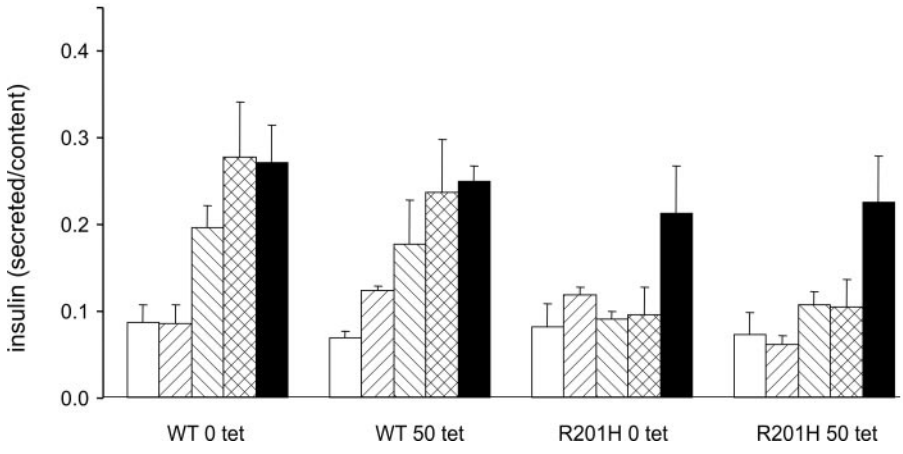

FIG. 6. Insulin secretion measured from INS-1 cells transfected with Kir6.2 or Kir6.2-R201H as indicated, in the absence of substrate (□) or in the presence of 1 mmol/l (▨), 5 mmol/l (▧), or 20 mmol/l (▩) methyl succinate or of 0.5 mmol/l tolbutamide plus 5 mmol/l methyl succinate (■). Data are normalized to insulin content to correct for differences in cell number or insulin content. Data are the means of three separate experiments. For each experiment, four replicates were performed for each concentration.

therefore consistent with the idea that the β-cells of patients carrying neonatal diabetes mutations remain hyperpolarized during challenge with metabolic substrates because of the reduced sensitivity of their K_{ATP} channels to block by ATP.

Relationship between K_{ATP} current and electrical activity. Our results show that there is a very steep relationship between K_{ATP} conductance and membrane potential. This arises because the membrane potential of the β-cell is determined almost entirely by the activity of K_{ATP} channels (30). There must also be a small inward current that depolarizes the β-cell when K_{ATP} channels are shut (31), but this has still not been identified. The inward current has little effect on membrane potential when K_{ATP} channels are open because it is electrically shunted (opposed) by the K_{ATP} current. However, when most K_{ATP} channels are closed, the input resistance of the β-cell becomes very high, and consequently tiny changes in current have large effects on membrane potential (31). Our data indicate that this occurs when the K_{ATP} conductance is less than ~0.3 nS, which suggests that the inward conductance must be of this magnitude.

The steep relationship between K_{ATP} conductance and membrane potential also explains why potentiators of insulin secretion, such as arginine and acetylcholine, only stimulate insulin secretion in the presence of glucose (31). These agents induce small inward currents in β-cells (<0.35 nS) (32,33), which in the absence of glucose are shunted by the much larger K_{ATP} conductance and thus have no effect on membrane potential. Only when K_{ATP} channels are largely closed by glucose and the conductance is very low (<0.35 nS) will they be able to influence the membrane potential. Our data predict that potentiators of insulin secretion will have no effect in neonatal diabetic patients because of the large K_{ATP} conductance. However, they will become effective when K_{ATP} channels are closed by sulfonylureas, such as tolbutamide and glibenclamide. A recent report indicates that this is indeed the case for patients with neonatal diabetes (34).

Tolbutamide block. We observed that tolbutamide produced somewhat less block of R201H and tetR201H channels than WT channels. A reduction in tolbutamide block was also observed for homomeric Kir6.2-R201H/SUR1 channels expressed in *Xenopus* oocytes (4). This can be explained by the fact that intracellular Mg-nucleotides enhance the block by sulfonylureas, through prevention of the stimulatory effects of Mg-nucleotides at SUR1 (35), and that some Mg-nucleotides are likely to remain in the whole-cell configuration. In the case of wild-type channels, this unmasks the inhibitory effect of Mg-nucleotides at Kir6.2, which adds to the block produced by sulfonylureas. This effect will be smaller for Kir6.2-R201H channels because they are less sensitive to Mg-nucleotide block. Tolbutamide at 0.5 mmol/l partially blocked R201H and tetR201H channels and also depolarized the β-cell membrane and induced action potentials, which is consistent with the observation that the drug was able to enhance insulin secretion from these cells.

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