Original Article

An ATP-Binding Mutation (G334D) in *KCNJ11* Is Associated With a Sulfonylurea-Insensitive Form of Developmental Delay, Epilepsy, and Neonatal Diabetes

Ricard Masia,[1] Joseph C. Koster,[1] Stefano Tumini,[2] Francesco Chiarelli,[2] Carlo Colombo,[3] Colin G. Nichols,[1] and Fabrizio Barbetti[3,4,5]

Mutations in the pancreatic ATP-sensitive K^+ channel (K_{ATP} channel) cause permanent neonatal diabetes mellitus (PNDM) in humans. All of the K_{ATP} channel mutations examined result in decreased ATP inhibition, which in turn is predicted to suppress insulin secretion. Here we describe a patient with severe PNDM, which includes developmental delay and epilepsy, in addition to neonatal diabetes (developmental delay, epilepsy, and neonatal diabetes [DEND]), due to a G334D mutation in the Kir6.2 subunit of K_{ATP} channel. The patient was wholly unresponsive to sulfonylurea therapy (up to 1.14 mg · kg^{-1} · day^{-1}) and remained insulin dependent. Consistent with the putative role of G334 as an ATP-binding residue, reconstituted homomeric and mixed WT+G334D channels exhibit absent or reduced ATP sensitivity but normal gating behavior in the absence of ATP. In disagreement with the sulfonylurea insensitivity of the affected patient, the G334D mutation has no effect on the sulfonylurea inhibition of reconstituted channels in excised patches. However, in macroscopic rubidium-efflux assays in intact cells, reconstituted mutant channels do exhibit a decreased, but still present, sulfonylurea response. The results demonstrate that ATP-binding site mutations can indeed cause DEND and suggest the possibility that sulfonylurea insensitivity of such patients may be a secondary reflection of the presence of DEND rather than a simple reflection of the underlying molecular basis.
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From the [1]Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri; the [2]Department of Pediatrics, University of Chieti, Chieti, Italy; [3]Bambino Gesù Children's Hospital IRCCS, Piazza S Onofrio 4, 00164 Rome, Italy; the [4]S. Raffaele Scientific Park Foundation, Rome, Italy; and the [5]Department of Internal Medicine, University of Tor Vergata, Rome, Italy.

Address correspondence and reprint requests to C.G. Nichols, Department of Cell Biology and Physiology, Washington University School of Medicine, 660 South Euclid Ave., St. Louis, MO 63110. E-mail: cnichols@wustl.edu. Or to F. Barbetti, S. Raffaele Scientific Park Foundation, Room B303, Via di Castelromano, 100, 00128, Rome, Italy. E-mail: fabrizio.barbetti@spr-r.it.

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DEND, developmental delay, epilepsy, and neonatal diabetes; K_{ATP} channel, ATP-sensitive K^+ channel; NDM, neonatal diabetes mellitus; PNDM, permanent NDM.

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Perturbations in electrical signaling in the β-cell are predicted to impair insulin release and result in inappropriate serum insulin levels for a given blood glucose concentration. Both animal and human studies now demonstrate that underexcitability can suppress insulin release and contribute to hyperglycemia and neonatal diabetes mellitus (NDM) in humans (1,2). Specifically, heterozygous activating mutations in the pancreatic ATP-sensitive K^+ channel (K_{ATP} channel), which result in suppression of β-cell electrical activity, underlie both permanent NDM (PNDM) and transient NDM (1,3–13). Importantly, sulfonylureas, which inhibit K_{ATP} channel, have proven an alternative treatment to life-long insulin injections in many patients with K_{ATP} channel–induced diabetes (5,14,15).

In low glucose, K_{ATP} channels provide the dominant membrane conductance, maintaining the β-cell in a hyperpolarized state. As [ATP]/[ADP] rises with increased glucose metabolism, K_{ATP} channels close, leading to membrane depolarization, voltage-driven Ca^{2+} entry, and Ca^{2+}-dependent insulin release. Thus, decreased responsiveness of K_{ATP} channel to elevated [ATP]/[ADP] should lead to decreased secretion and a diabetic phenotype. Structurally, K_{ATP} channels are hetero-octomers, consisting of four subunits each of the pore-forming Kir6.2 subunit (*KCNJ11*) and four regulatory sulfonylurea receptor subunits (SUR1, *ABCC8*) (16–18). ATP inhibits the channel by directly interacting with Kir6.2 (19–23), where binding of ATP to one of the four subunits is sufficient to close the channel (24,25). SUR1 mediates stimulation of channel activity by Mg nucleotides (26–29) and inhibition by sulfonylurea compounds (19).

To date, >30 heterozygous, activating mutations in K_{ATP} channels have been reported (14,30,31). With few exceptions (11,12), mutations localize to the Kir6.2 subunit, and in vitro analyses demonstrate that they activate channel activity by decreasing sensitivity to inhibitory ATP (7,8,12,32–37), either by directly decreasing ATP binding or by altering intrinsic channel gating, i.e., increasing open probability in the absence of ATP (*P*o,zero) and, thereby, allosterically altering ATP sensitivity. Convincing evidence exists for a genotype-phenotype relationship in which the severity of the mutation correlates with the severity of the disease: at one end of the spectrum, strongly activating mutations underlie developmental delay, epilepsy, and neonatal diabetes (DEND), which likely reflect K_{ATP} channel overactivity in brain, skeletal muscle, and nerve, while

at the other end mildly activating mutations underlie transient NDM with no neurological features. In heterozygous expression, most channels consist of a mixture of both wild-type and mutant Kir6.2 subunits. Since occupancy of only one binding site is sufficient for channel closure (24,25), only channels composed of four mutant subunits will exhibit significant loss of ATP sensitivity. Conversely, in the case of gating mutants the effects of the mutation are transmitted to the entire pore; thus, ATP sensitivity is decreased for all channel species that contain mutant subunits (7,9,32). It was therefore originally postulated that ATP-binding mutations should underlie milder forms of NDM and that gating mutations should underlie the more severe forms (32,33). However, there are now reports of gating mutations that cause neonatal diabetes but no neurological effects (34), as well as a report of a mutation in the ATP-binding site (R50P) that causes DEND syndrome (36).

Several clinical studies (1,3,5,9,14,15,38) of patients with K_{ATP} channel–induced NDM have now shown that sulfonylureas alone are effective in maintaining glycemic control over a period of months or longer. However, in many cases the required sulfonylurea dose is significantly higher than that normally used to treat type 2 diabetes (14,15). This difference in dose may be accounted for by the mechanism of K_{ATP} channel overactivity. In recombinant expression, gating mutations in K_{ATP} channel concomitantly decrease high-affinity sulfonylurea block. Conversely, ATP-binding site mutations decrease ATP sensitivity without altering sulfonylurea sensitivity (32,37,39,40); therefore, patients bearing such mutations may be expected to exhibit normal drug sensitivity.

Here we describe a proband with a novel heterozygous activating mutation (G334D) in *KCNJ11*. Previous studies had identified G334 (21,24) and the neighboring F333 residue (41,42) as significant contributors to the ATP-binding site. The G334D mutation causes severe loss of ATP sensitivity but without discernable effects on ATP-independent gating. Rather than a mild form of the disease, the patient was diagnosed with NDM soon after birth and exhibits the extra-pancreatic symptoms of DEND. In addition, even though G334D does not alter sulfonylurea sensitivity in vitro, the patient is unresponsive to sulfonylurea therapy ($1.14\ mg \cdot kg^{-1} \cdot day^{-1}$) and remains on insulin. This is the second ATP-binding site mutation that gives rise to the most severe form of NDM. The responsiveness of the mutation to sulfonylurea in recombinant channels suggests that the severity of the disease itself may be a determinant of in vivo sulfonylurea responsiveness.

RESEARCH DESIGN AND METHODS

Genetics and molecular biology. The intronless *KCNJ11* gene was amplified in three overlapping fragments by PCR and directly sequenced (4). The G334D mutation was identified in the proband and not in the parents; thus, the mutation arose spontaneously, as in most cases of PNDM. Informed consent was obtained in order to initiate a sulfonylurea trial. G334D was engineered into mouse Kir6.2 cDNA using the Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by direct sequencing. G334D was combined with another mutation (N160E) that endows the channel with strong inward rectification without significantly altering gating properties (43). This manipulation allows determination of zero current, independently of ATP sensitivity, by blocking the channel at positive voltages with polyamines (Fig. 2). Thus, in the patch-clamp experiments described here, we used Kir6.2[N160E] as the appropriate control (referred to as WT in this study) with which to compare Kir6.2[N160E, G334D] (referred to as G334D here).

Expression of K_{ATP} channels in COSm6 cells. COSm6 cells were transfected with cDNA using FuGENE 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN). Total DNA (0.3 μg mouse Kir6.2 [GenBank no. D50581]) (44) plus 0.5 μg hamster SUR1 (GenBank no. L40623) (27) plus 0.3 μg green fluorescence protein as a marker for transfection was mixed with FuGENE 6 and preincubated for 1 h. Cells were incubated in the presence of the transfection mixture for 12–24 h and then plated on sterile glass coverslips overnight before patch-clamp experiments. To simulate the heterozygous state, cells were transfected with equal amounts of WT and G334D DNA.

Electrophysiological methods. Patch-clamp experiments were performed at room temperature on transfected COSm6 cells. Membrane patches were voltage-clamped using an Axopatch 1-D amplifier. All currents were measured at a membrane potential of −50 mV (pipette voltage +50 mV). Bath and pipette control solutions (K_{INT}) contained: 150 mmol/l KCl, 10 mmol/l HEPES, and 1 mmol/l EGTA (pH 7.4). ATP and ADP were added to the bathing solution as dipotassium salts. Where indicated, $MgCl_2$ was added to the bathing solution to a calculated $[Mg^{2+}_{free}]$ of 0.5 mmol/l. Tolbutamide was dissolved in K_{INT} from a 100 mmol/l stock solution in 100 mmol/l KOH. Spermine (Sigma, St. Louis, MO) was dissolved in K_{INT}. Data were collected using the pClamp 8.2 software suite (Axon Instruments, Union City, CA) and Microsoft Excel.

Data analysis. The ATP dose-response of WT and mixed WT+G334D channels (in either the presence or absence of Mg^{2+}) was empirically quantified by fitting the raw data with a Hill equation plus an offset:

$$Irel = f + \frac{1-f}{1+\left(\frac{\mathrm{ATP}}{k_{1/2}}\right)^{H}} \quad (1)$$

where *Irel* is the current relative to that in the absence of ATP, $k_{1/2}$ is the half-maximal inhibitory ATP, H is the Hill coefficient, and f is the fraction of unblocked channels at saturating ATP (24,36). The stimulation of homomeric G334D by MgATP was quantified by fitting with the following modified Hill equation:

$$Irel = 1 + A^{*}\left(1 - \frac{1}{1+\left(\frac{\mathrm{MgATP}}{k_{1/2}}\right)^{H}}\right) \quad (2)$$

where Irel is the current relative to that in the absence of MgATP, $k_{1/2}$ is the half-maximal stimulatory MgATP, H is the Hill coefficient, and A is the maximal increase in Irel due to MgATP stimulation.

The ATP dose-response of mixed WT+G334D channels was also fitted with a model assuming a binomial distribution of mutant and nonmutant subunits, free association of both subunit types into channel tetramers, and binding of ATP to a single nonmutant subunit to be sufficient for closure of the channel (24). The frequency P_k of each channel species was calculated with the following equation:

$$P_k = \binom{4}{k} \cdot P^{k} \cdot (1-P)^{4-k} \quad (3)$$

where k is the number of nonmutant subunits in a given channel species and P is the probability of incorporation of a nonmutant subunit into each of the four positions in the channel tetramer. (To account for experimental deviations in the DNA transfection ratio, we determined the actual transfection ratio by fitting the ATP dose-response of mixed WT+G334D channels.) The activity of each species in the presence of ATP was calculated from the following equation:

$$\sum_{k=0}^{4} P_k \cdot [1-(1-b_{wt})^{k}(1-b_{m})^{(4-k)}] \quad (4)$$

where b_{wt} and b_m are the probability of ATP binding to the WT or mutant subunit, respectively, and $4-k$ is the number of mutant subunits in the channel species. The probabilities b_{wt} and b_m are assumed to be equal to the closed probability in the respective homomeric channels (assuming a simplified two-state model with ATP binding to the closed state).

*P*o,zero was estimated from stationary fluctuation analysis of macroscopic currents (45) on short (<1 s) recordings of currents in zero ATP and in 10 mmol/l ATP or 10 μmol/l spermine (for estimation of ATP-independent noise). Currents (corresponding to ~100–1,000 channels) were filtered at 1 kHz and digitized at 3 kHz with 12-bit resolution. Mean patch current (I) and variance (σ^2) in the absence of ATP were obtained by subtraction of the mean current and variance in 10 mmol/l ATP or 10 μmol/l spermine (i.e., assuming all channels fully closed or blocked). Single channel current (i) was assumed to be −3.75 pA at −50 mV, corresponding to WT single-channel conductance of 75 pS (43). *P*o,zero was then estimated from the following equation:

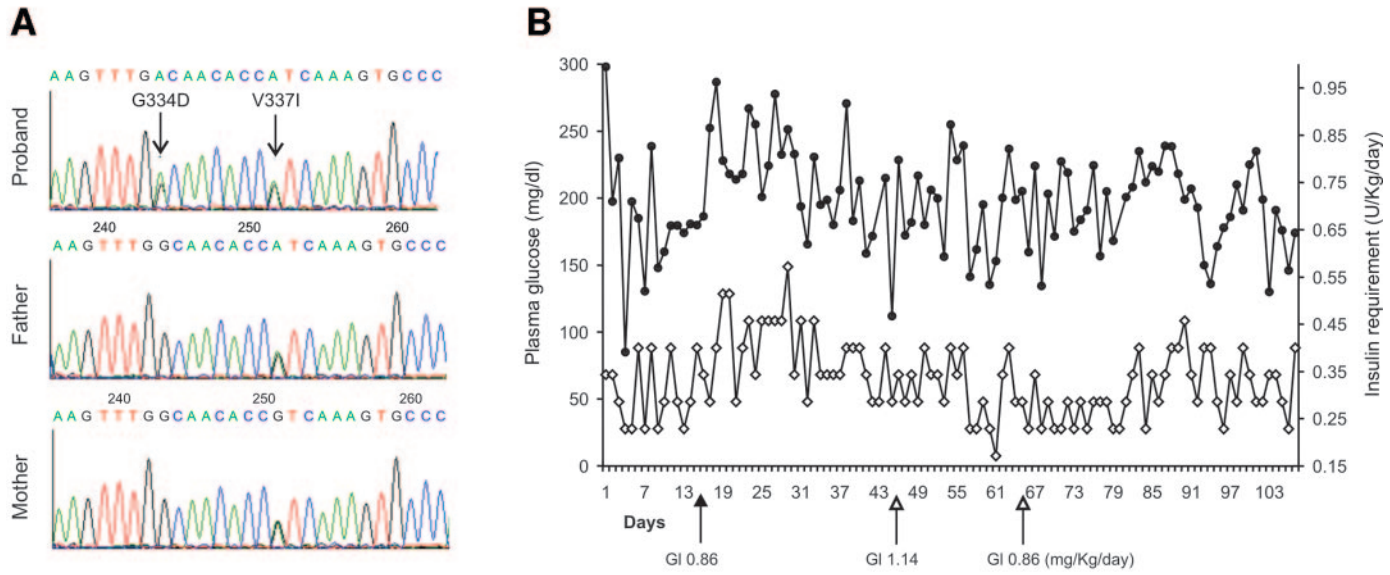

FIG. 1. Sulfonylurea trial in a patient with the heterozygous KCNJ11 mutation G334D. *A*: Sequence chromatogram of G334D carrier (*top*), unaffected father (*middle*), and unaffected mother (*bottom*). Note that all three are heterozygous for the V337I polymorphism, but only the patient is heterozygous for G334D. *B*: Blood glucose values (●) vs. insulin dose (◇) measured daily over a period of 106 days in the G334D patient. Glibenclamide was administered daily starting at day 15 (solid arrow) and the dosing regiment was changed as indicated (open arrows).

$$Po,\text{zero} = 1 - \left(\frac{\sigma^2}{i \cdot I}\right) \quad (5)$$

The tolbutamide dose-response was quantified by fitting the data with a double-Hill equation:

$$Irel = \left((1-f) + \frac{f}{1 + \left(\frac{\text{tolb}}{K_{1/2,A}}\right)^{H_A}}\right) * \left(\frac{1}{1 + \left(\frac{\text{tolb}}{K_{1/2,B}}\right)^{H_B}}\right) \quad (6)$$

where f is the fraction of high-affinity block (46), $K_{1/2}$ is the half-maximal inhibitory tolbutamide concentration for either the high-affinity ($K_{1/2,A}$) or low-affinity site ($K_{1/2,B}$), and H is the Hill coefficient for either the high-affinity (H_A) or low-affinity site (H_B).

Macroscopic $^{86}Rb^+$ efflux assays. COSm6 cells in 12-well plates were incubated for 24 h in culture medium containing ^{86}RbCl (1 μCi/ml) 2 days after transfection. Before measurement of $^{86}Rb^+$ efflux, cells were washed twice with Ringer's solution (118 mmol/l NaCl, 2.5 mmol/l $CaCl_2$, 1.2 mmol/l KH_2PO_4, 4.7 mmol/l KCl, 25 mmol/l $NaHCO_3$, 1.2 mmol/l $MgSO_4$, and mmol/l 10 HEPES; pH 7.4) or Ringer's solution plus metabolic inhibition (1 mmol/l 2-deoxy-D-glucose and 2.5 μg/ml oligomycin). At selected time points, the solution was aspirated from the cells and replaced with fresh solution; after completion of the assay, cells were lysed with 1% SDS and aspirated. Aspirated samples were assayed in a scintillation solution. Raw data are shown as $^{86}Rb^+$ efflux relative to total counts (including all time points and the lysate for each construct). The rate constant (k_1) and amplitude (A_1) of nonspecific $^{86}Rb^+$ efflux was obtained from untransfected cells by fitting the data with a single-exponential equation:

$$\text{Flux} = A_1 \cdot [1 - \exp(-k_1 \cdot t)] \quad (7)$$

The rate constant of K_{ATP} channel–specific $^{86}Rb^+$ efflux (k_2) was obtained by fitting the data with a double-exponential equation:

$$\text{Flux} = A_1 \cdot [1 - \exp(-k_1 \cdot t)] + (1 - A_1) \cdot [1 - \exp(-k_2 \cdot t)] \quad (8)$$

where values for k_1 and A_1 were obtained from untransfected cells. The glibenclamide dose-response was quantified by fitting the data with a Hill equation.

Results are presented as means ± SEM. Statistical tests and P values are noted in figure legends where appropriate.

RESULTS

Genotyping and clinical description of G334D patient. A 14-year-old male patient diagnosed with DEND was found to have a de novo G334D mutation in one of the Kir6.2 alleles (Fig. 1*A*). The patient was born at 41 weeks after an uneventful pregnancy (although intrauterine developmental delay of ~2 weeks was detected by ultrasound at 31 weeks' gestation), with a birth weight of 2,440 g (<3rd centile). On day 2 after birth, the patient was transferred to the local neonatal intensive care unit for sepsis (blood culture positive for *Staphylococcus gallinarum*, normal cerebrospinal fluid parameters by lumbar puncture, and negative liquor culture), where he presented with mild hyperglycemia (8.3 mmol/l). At 14 days of life, a more pronounced hyperglycemia (13.8 mmol/l, pH 7.31) was detected and treated with insulin (0.2–0.4 IU · kg^{-1} · day^{-1}). C-peptide was not measurable, and type 1 diabetes autoantibodies were absent. Abdominal ultrasonogram showed a normal pancreas. At physical examination, somatic developmental delay (length and weight significantly below the 3rd percentile for the corresponding age) with mild dysmorphic features (downturned mouth, low-set ears) was present, and neurological examination showed severe hypotonia of all four limbs. At 4 months of age, the patient showed myoclonic seizures with multifocal sharp waves and slow delta waves on electrocardiogram. All features are consistent with complete DEND syndrome. At 14 months, Brunet-Lezine scoring showed a developmental age of 1 month. Nuclear magnetic resonance of the brain at 1 year was normal, although "thickening" of the gray matter of the temporal lobes was noted. Nuclear magnetic resonance of the central nervous system (at age 14 years) confirmed the previous unremarkable findings.

Notably, an attempt to progressively wean the patient onto glibenclamide (dissolved in water at a concentration of 5 mg/ml) (15) was unsuccessful, with C-peptide levels (radioimmunoassay with a commercial kit) undetectable during the entire trial (>90 days with glibenclamide dose of 0.8 mg · kg^{-1} · day^{-1} or higher; Fig. 1*B*). Neurological symptoms were not alleviated during the oral sulfonylurea treatment, and at 14 years the patient weighs 18 kg, remains bedridden, and does not speak or communicate; he is on continuous therapy for epilepsy (valproic acid, 33.3 mg · kg^{-1} · day^{-1}).

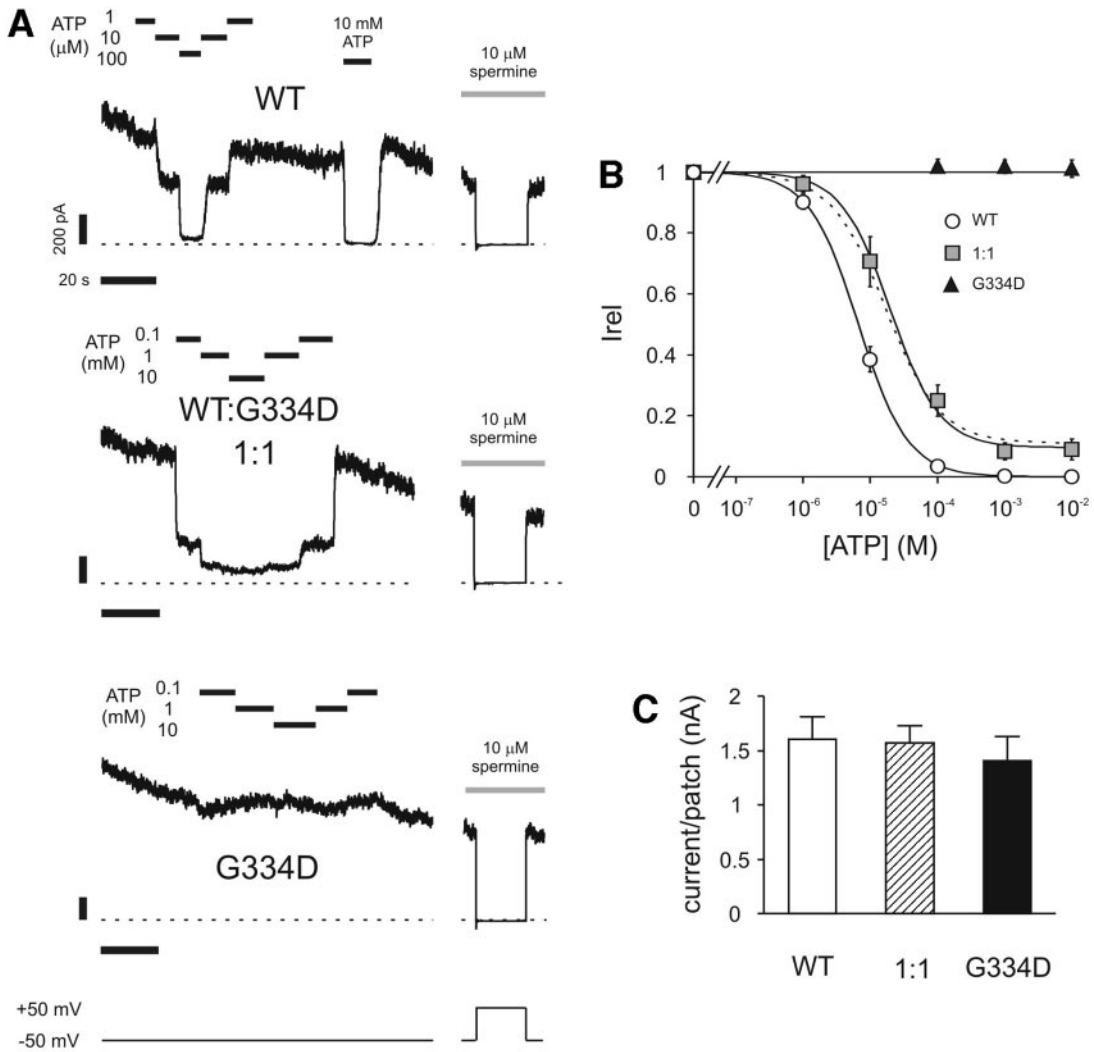

FIG. 2. Reduced ATP sensitivity of reconstituted K_{ATP} channels containing G334D subunits. *A*: Representative currents recorded from inside-out membrane patches from COS cells expressing wild-type, mixed WT+G334D, or homomeric G334D-containing K_{ATP} channels at −50 mV in K_{int} solution (see RESEARCH DESIGN AND METHODS). Patches were exposed to differing ATP, and baseline current was determined by addition of either ATP (10 mmol/l) or spermine (10 μmol/l at +50 mV). *B*: Steady-state dependence of membrane current on ATP (relative to current in zero ATP [Irel]) for wild-type and G334D-containing channels. Data points represent the means ± SEM (n = 8–15 patches). The solid lines correspond to least squares fits of a Hill equation (see RESEARCH DESIGN AND METHODS, Table 1). The dotted line corresponds to a Markworth model fit (24) of mixed WT+G334D channels (see RESEARCH DESIGN AND METHODS). *C*: Maximal current density (nA/patch) in the absence of ATP from membrane patches expressing WT, mixed WT+G334D, or homomeric G334D channels (n = 29–46 patches).

G334D channels are insensitive to ATP inhibition. Coexpression of WT Kir6.2 and SUR1 in COSm6 cells leads to expression of K_{ATP} channels that are inhibited by ATP in excised patch-clamp experiments with an ATP sensitivity similar to native channels ($K_{1/2}$ = 6.7 μmol/l, Fig. 1) (44). Conversely, channels containing the G334D mutation are extremely insensitive to ATP inhibition, with no inhibition up to 10 mmol/l ATP (Fig. 2). Stationary noise analysis indicates that the open probability (*P*o,zero) of G334D channels is not different from WT (0.35 ± 0.04 vs. 0.27 ± 0.02 for WT, n = 13 and 27 patches, respectively). These results are consistent with previous electrophysiological characterizations of G334D (21,24) and indicate that G334D exerts its effects by directly preventing ATP binding to the cytoplasmic domain of Kir6.2, rather than by indirectly altering ATP sensitivity by changing the *P*o,zero.

To characterize the electrophysiological properties of heterozygous G334D channels, COSm6 cells were transfected with a one-to-one mixture of WT and G334D cDNA. This approach is expected to recapitulate the properties of native channels from PNDM patients with heterozygous Kir6.2 mutations. Mixed WT+G334D channels are less ATP sensitive than homomeric WT channels ($K_{1/2}$ = 19.9 vs. 6.7 μmol/l for WT, Fig. 2*A* and *B*), and, notably, they exhibit a current plateau at high ATP concentrations (≥1 mmol/l ATP). This plateau (~9.6%) corresponds to the expected fraction of channels (1/16) composed solely of G334D subunits, based on a binomial distribution of subunits (24).

Tolbutamide sensitivity of G334D channels is unaltered in inside-out membrane patches. We explored the potential mechanism underlying sulfonylurea insensitivity of the G334D patient by assessing sulfonylurea sensitivity of homomeric G334D and mixed WT+G334D channels in excised membrane patches. All three (homomeric WT, mixed WT+G334D, and homomeric G334D) are equally well inhibited by tolbutamide at both high- (IC_{50} = ~2 μmol/l) and low-affinity (IC_{50} = ~6 mmol/l) components of block (Fig. 3 and Table 1). These data indicate that the G334D mutation does not directly alter sulfonylurea binding to K_{ATP} channels.

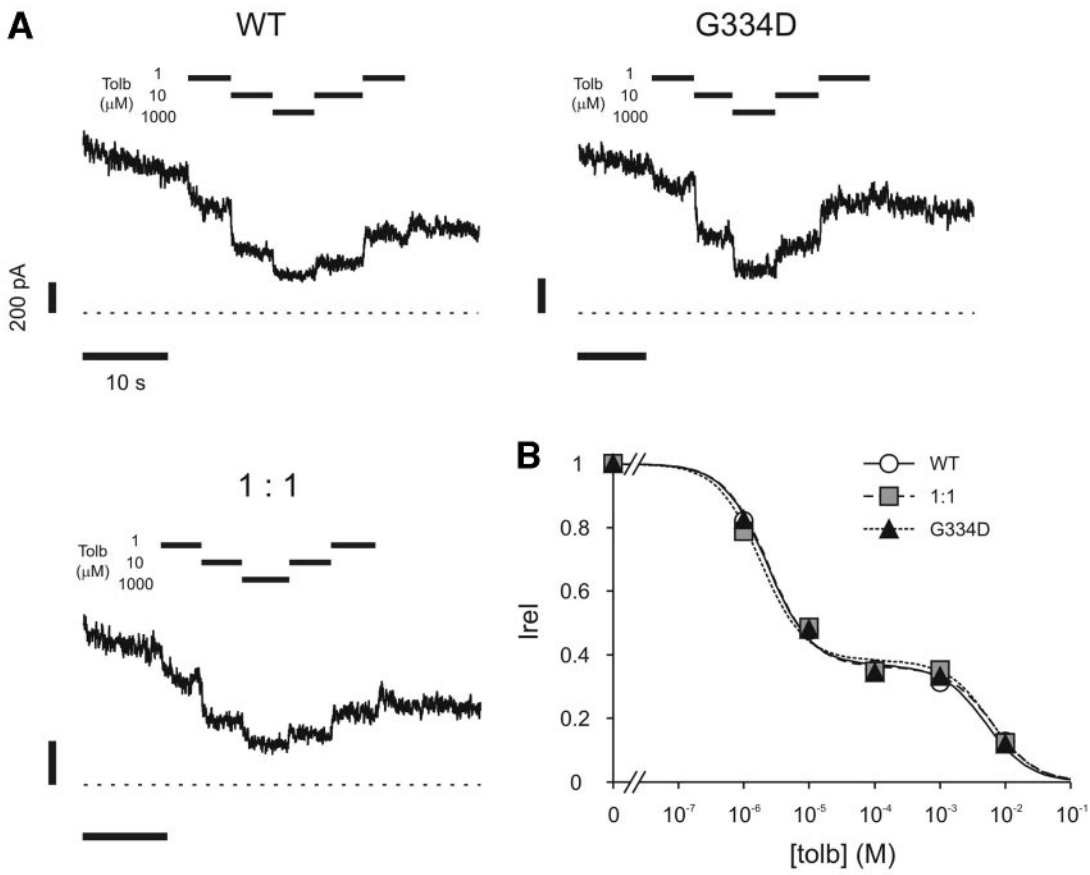

FIG. 3. Unaltered tolbutamide sensitivity of K_{ATP} channel currents in membrane patches from COS cells expressing G334D-containing channels. *A*: Representative currents recorded from inside-out membrane patches from COS cells expressing WT, mixed WT+G334D, or homomeric G334D channels at −50 mV. Patches were exposed to differing tolbutamide, and baseline current was determined by addition of either ATP (10 mmol/l) or spermine (10 μmol/l at +50 mV). *B*: Steady-state dependence of membrane current on tolbutamide (relative to current in zero tolbutamide [Irel]) for WT and mutant channels. Data points represent the means ± SEM (n = 18–21 patches). Data were fit with a double Hill equation (see RESEARCH DESIGN AND METHODS, Table 1).

Overactivity of mixed WT+G334D channels in intact cells. The results are consistent with the previously observed properties of K_{ATP} channels formed from Kir6.2 subunits with ATP-binding site mutations: Although homomeric channels are very ATP insensitive, ATP sensitivity of mixed WT+G334D channels is only mildly reduced, and ATP-binding site mutations have no effect on the intrinsic sensitivity to sulfonylureas. These results are not, however, consistent with the observed disease phenotype resulting from the G334D mutation, a severe diabetes that is refractory to sulfonylureas. To obtain further insight, we examined channel activity by measuring macroscopic $^{86}Rb^+$ fluxes in intact cells (37). Mixed WT+G334D channels are significantly more active than WT under basal conditions (Fig. 4). Moreover, when metabolic inhibition is applied, WT activity increases significantly, but both homomeric G334D and mixed WT+G334D channel activities are already maximal (Fig. 4*B* and *C*). Thus, even though mixed WT+G334D channels exhibit minimal shift in intrinsic ATP sensitivity (Fig. 2*B*), they are maximally active on cells. We next assessed on-cell patch current (relative to maximal current following excision) in intact cells (Fig. 5*A*), and, again, both mixed WT+G334D and homomeric G334D channels are significantly more active than WT, even though expression levels are similar (Fig. 2*C*). Unexpectedly, in metabolic inhibition, homomeric G334D channels exhibited lower fluxes than WT or mixed WT+G334D channels. Since homomeric G334D channel density in

TABLE 1
Properties of G334D-containing channels

	WT	1:1	G334D
ATP sensitivity*			
f	1.0	0.90	0
$K_{1/2}$ (μmol/l)	6.7	20.0	NA
n	15	12	8
Tolbutamide sensitivity in excised patches			
High affinity†			
f	0.62	0.64	0.63
$K_{1/2}$ (μmol/l)	1.8	2.4	2.3
Low affinity			
$K_{1/2}$ (mmol/l)	6.0	6.5	5.1
n	21	18	21
Glibenclamide sensitivity in intact cells‡			
Basal			
$K_{1/2}$ (nmol/l)	4.1	8.3	32.1
Metabolic inhibition			
$K_{1/2}$ (nmol/l)	3.1	4.4	50.5
n	5	5	5

n = number of patches (for ATP sensitivity and tolbutamide sensitivity) or number of $^{86}Rb^+$ flux experiments (for glibenclamide sensitivity). *f = fraction of channels blocked at 10 mmol/l ATP. †f = fraction of high-affinity block. ‡glibenclamide block from $^{86}Rb^+$ flux experiments in the presence or absence (basal) of metabolic inhibition.

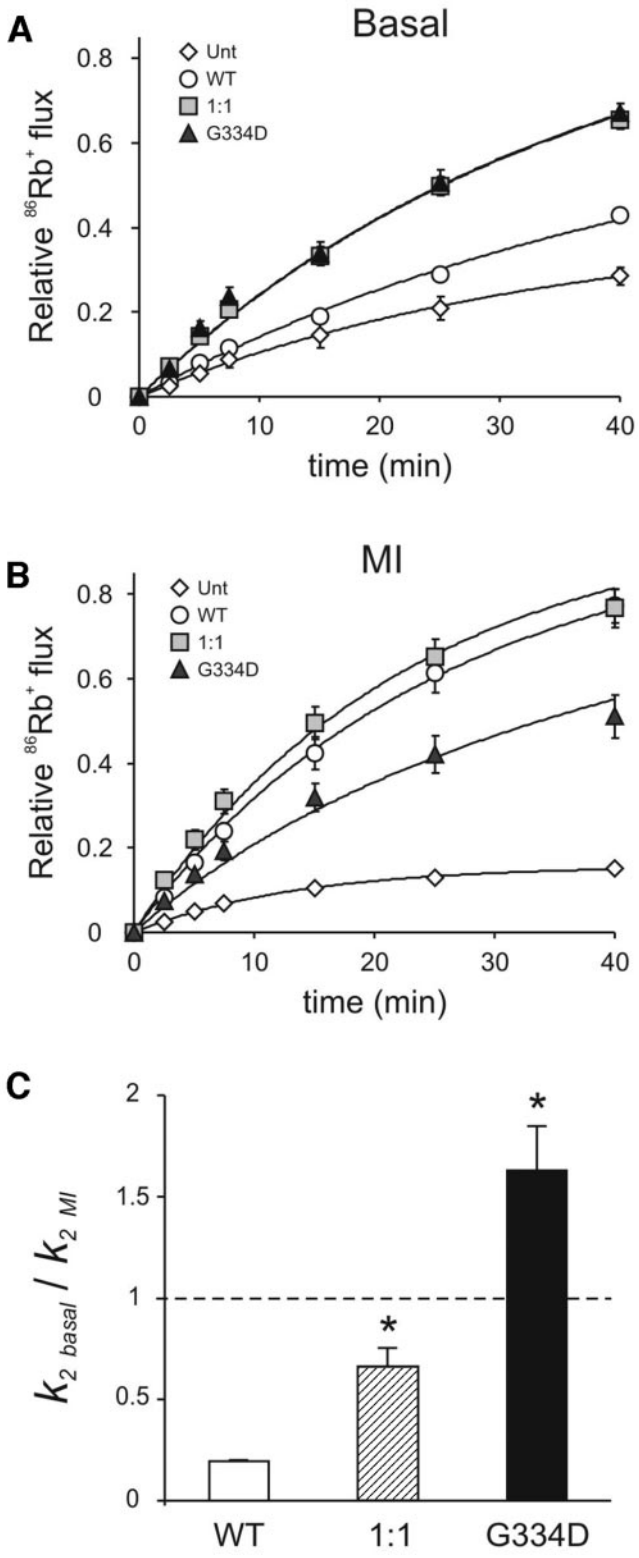

FIG. 4. Increased basal activity in intact cells expressing G334D-containing K_{ATP} channels. Relative efflux of $^{86}Rb^+$ as a function of time in basal conditions (*A*) or in the presence of metabolic inhibition (*B*) for reconstituted wild-type and mutant K_{ATP} channels and untransfected controls. All transfections were done in parallel. Graphs show compiled data (means ± SEM) from five experiments. Data were fit with a double-exponential equation to obtain rate constants for K_{ATP} channel–dependent efflux, k_2 (see RESEARCH DESIGN AND METHODS). *C*: Rate constants for K_{ATP} channel–dependent $^{86}Rb^+$ efflux (k_2) in basal conditions relative to metabolic inhibition (MI) for WT, mixed WT+G334D, and homomeric K_{ATP} channels (n = 5 experiments). *P < 0.05 as compared with WT by paired Student's t test.

isolated membrane patches is not significantly different from that of WT or mixed WT+G334D channels (Fig. 2*C*), we speculate that the persistent high K^+ conductance in cells expressing homomeric G334D channels may slow cell growth and thus lead to decreased fluxes in intact cells.

Importantly, the on-cell activity of mixed WT+G334D channels cannot be accounted for solely by the fraction of ATP-insensitive (i.e., homomeric G334D) channels in the mixture (~40% on-cell activity in Fig. 5*A* vs. ~10% current plateau in Fig. 2*B*). Mg nucleotides can significantly control channel activity both through phosphorylation and interactions with the SUR1 subunits (47) and have been shown to preferentially stimulate hyperactive Kir6.2 mutations (36,48). WT channels are strongly inhibited by ATP in Mg-containing solutions (Fig. 5*B*), but mixed WT+G334D channels are less effectively inhibited, such that a plateau of ~40% activity remains even at very high ATP (Fig. 5*B*). Homomeric G334D channels, lacking any ATP inhibitory effect, are strongly stimulated by MgATP (Fig. 5*B*). Furthermore, when the effect of MgADP was examined (0.5 mmol/l MgADP in the presence of 0.1 mmol/l MgATP), there was a stronger stimulation of mixed WT+G334D than WT channels (Fig. 5*C* and *D*). Thus, mixed WT+G334D channels show a greatly enhanced response to Mg nucleotide stimulation.

Glibenclamide sensitivity is reduced in G334D channels in the intact cell. That the G334D patient is refractory to sulfonylurea therapy despite the unchanged sulfonylurea sensitivity of G334D channels in excised patches (Figs. 1 and 3) suggests a discrepancy between channel behavior in the native context and in excised patches. To assess sulfonylurea sensitivity in the intact cell, macroscopic $^{86}Rb^+$ efflux was measured in the presence and absence of metabolic inhibition. In both control and metabolic inhibition, G334D-containing channels are less sensitive to glibenclamide than WT (Fig. 6, Table 1). While the results demonstrate slight reduction of sulfonylurea sensitivity in intact cells, this reduction is unlikely to account for the complete refractoriness of the patient to sulfonylureas.

DISCUSSION

Unpredicted consequences of the G334D mutation. Previous studies demonstrated that G334D in Kir6.2 causes significant loss of ATP sensitivity of recombinant K_{ATP} channels due to alteration of the ATP binding site (21,24,49). We have now identified this mutation in a patient exhibiting complete DEND syndrome, unresponsive to sulfonylurea therapy. Together with R50P (36), this mutation confirms that ATP-binding site mutations can cause the most severe form of NDM.

The lack of sulfonylurea sensitivity is at odds with the previous prediction that, since ATP-binding site mutations do not affect intrinsic sulfonylurea sensitivity (39,40), carriers of such mutations should respond to sulfonylurea therapy (37). In agreement with previous experiments (24), homomeric G334D channels were completely insensitive to block by ATP at concentrations as high as 10 mmol/l ATP (Fig. 2). The *P*o,zero of G334D channels is not significantly different from WT, consistent with the effect of the mutation being on ATP binding rather than an indirect consequence of altered channel gating. Since ~94% of the tetrameric channels formed by coexpression of WT and mutant subunits will contain at least one WT

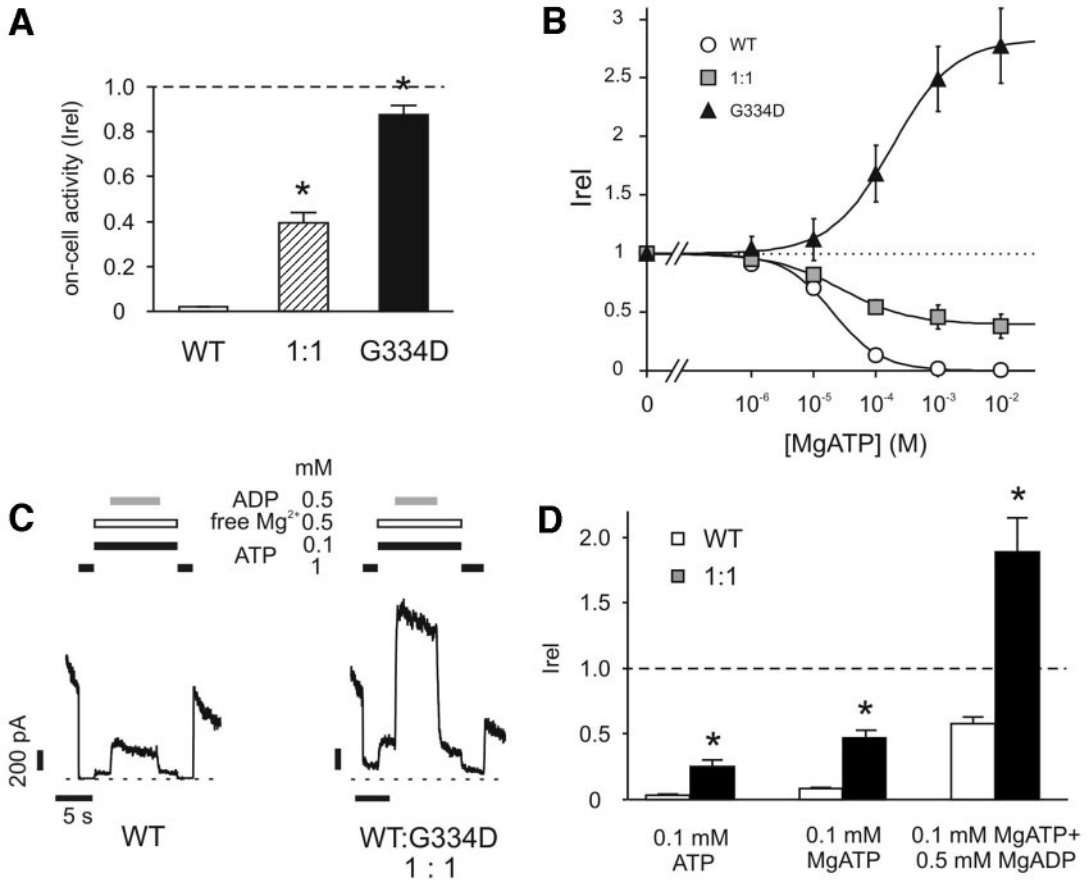

FIG. 5. Increased sensitivity to Mg nucleotides of K_{ATP} channels containing G334D subunits. *A*: On-cell current, relative to maximal postexcision current, from membrane patches expressing WT, heteromeric, or homomeric G334D channels (n = 29–47 patches). *$P < 0.05$ as compared with WT by paired Student's t test. *B*: Steady-state dependence of membrane current on ATP (relative to current in zero ATP [Irel]) in the presence of 0.5 mmol/l free Mg^{2+} for WT and G334D-containing channels. Data points represent the means ± SEM (n = 9–14 patches). The fitted lines correspond to least squares fits of modified Hill equations (see RESEARCH DESIGN AND METHODS) with the following H and $K_{1/2}$ values: WT, 1.10 and 20.3 μmol/l ATP; mixed WT+G334D, 0.79 and 28.0 μmol/l ATP; and homomeric G334D, 0.88 and 182.7 μmol/l ATP. *C*: Representative currents recorded from inside-out membrane patches containing WT or mixed WT+G334D channels at −50 mV in K_{INT} solution (see RESEARCH DESIGN AND METHODS). Patches were exposed to ATP, ADP, and Mg^{2+}, as indicated, and baseline current was determined by addition of either ATP (10 mmol/l) or spermine (10 μmol/l at +50 mV). *D*: Average current in Mg nucleotides, relative to control (zero nucleotides), from membrane patches expressing WT or mixed WT+G334D channels (n = 12–19 patches). *$P < 0.05$ as compared with WT by paired Student's t test.

subunit (based on binomial distribution), and since ATP binding to a single WT subunit is sufficient to effectively close the channel (24,25), the reduction in ATP sensitivity of mixed WT+G334D channels is modest (Fig. 2*B*). However, the remaining ~6% of the channels will be composed exclusively of G334D subunits and remain unblocked even at high ATP, yielding a current plateau in physiologic ATP (Fig. 2*B*). Still, this plateau of current is clearly insufficient to account for the level of activity of mixed WT+G334D channels in the intact cell (Fig. 5*A*). Rather, the increased activity in vivo appears to reflect a greater stimulatory effect of Mg nucleotides on G334D-containing heteromeric

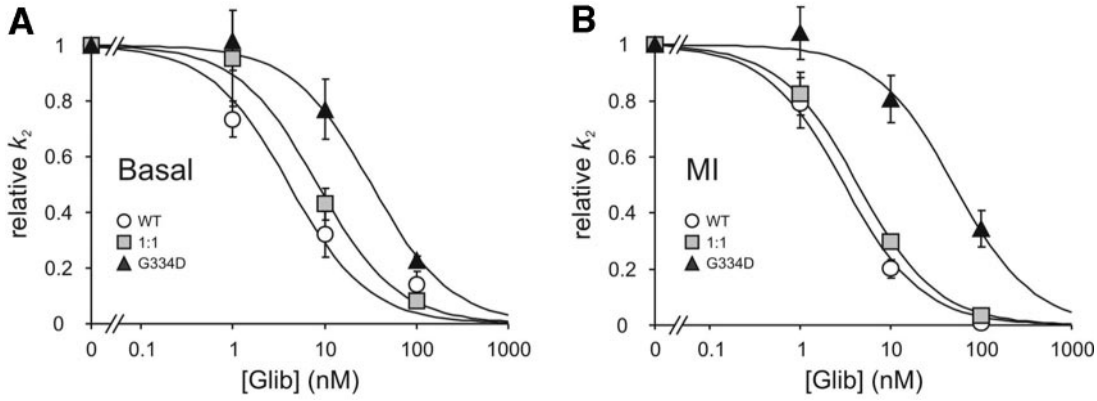

FIG. 6. Decreased glibenclamide sensitivity in intact cells expressing G334D-containing K_{ATP} channels. Steady-state dependence of $^{86}Rb^+$ efflux on glibenclamide (relative to zero glibenclamide) for WT and G334D-containing channels in basal conditions (*A*) and metabolic inhibition (*B*). Data points represent the means ± SEM (n = 5 experiments). Data were fit with a Hill equation (see RESEARCH DESIGN AND METHODS, Table 1).

channels (Fig. 5*B* and *C*). Enhanced Mg nucleotide stimulation of several NDM mutations has been noted previously and invoked as a critical contributor to the phenotype (30,34,36,48), but such a result for an ATP-binding site mutation is not trivially expected from existing models of channel gating (47), in which ATP inhibition and Mg nucleotide stimulation result from kinetically distinct processes. Nevertheless, such behavior might be expected if there is a state dependence to nucleotide stimulation, for example if Mg nucleotide interaction with SUR1 is enhanced when ATP is not bound by Kir6.2. By way of example, PIP2 activation and Mg uridine 5′-diphosphate activation of the K_{ATP} channel both appear to be dependent on the relative occupancy of the nucleotide-bound state (47,48,50).

Sulfonylurea resistance in the G334D patient. The proband was completely unresponsive to sulfonylurea treatment, and neither insulin dose, A1C, nor circulating C-peptide levels were affected over 20 days at a glibenclamide dose of 1.14 $mg \cdot kg^{-1} \cdot day^{-1}$, a concentration in excess of the typical range used to successfully treat PNDM patients with K_{ATP} channel mutations (14,15). This clinical observation is at odds with the expected effects of ATP-binding site mutations on sulfonylurea sensitivity; however, the present data reveal an important feature of sulfonylurea sensitivity. While the sulfonylurea sensitivity of G334D-containing channels in excised patches is not different from WT, sulfonylurea sensitivity is significantly lower in intact cells for homomeric G334D channels (Fig. 6). Of the cellular factors that may modulate the sulfonylurea response in vivo, Mg nucleotides are likely candidates: nucleotides both stimulate K_{ATP} channels through the SUR1 subunit (Mg-bound form) and inhibit activity through the Kir6.2 subunit (Mg-free form). Importantly, sulfonylurea block is more efficient in the presence of Mg nucleotides (46), possibly because sulfonylureas act by preventing Mg nucleotide stimulation of the channel through SUR1, thereby unmasking nucleotide block through Kir6.2. If ATP inhibition through Kir6.2 is absent or reduced, as in G334D channels, there may be a shift in apparent sulfonylurea sensitivity in the intact cell, partly explaining why higher doses of sulfonylureas are often required to treat patients with activating K_{ATP} channel mutations. In the present case, the modest shifts in sulfonylurea sensitivity associated with homomeric and heteromeric G334D channels are unlikely to account for the sulfonylurea unresponsiveness in the G334D patient, and secondary complications of the disease may be responsible (see below).

Sulfonylurea resistance with age in patients with Kir6.2 activating mutations. On average, DEND patients are less likely to respond to sulfonylurea therapy, and the patient response to sulfonylurea therapy tends to correlate with the severity of the disease (14). A progressive loss of β-cell mass could potentially explain why some DEND patients remain sulfonylurea unresponsive. The G334D patient described here received sulfonylurea treatment at age 14 years, and, interestingly, the median age at initiation of treatment is significantly higher for patients that do not respond to sulfonylurea therapy than for patients that do respond: 6 years of age for responders versus 18 years of age for nonresponders (14). Moreover, in two families (14), affected children were responsive to sulfonylurea therapy, whereas the affected mothers were not. This may also reflect an age dependency of sulfonylurea response, which could be explained by a progressive and irreversible loss of β-cell mass to a point where sulfonylureas are ineffective. In mouse models of neonatal diabetes due to K_{ATP} channel overactivity, we have observed progressive loss of β-cells and disruption of the islet architecture (2,13). Although the etiology is unclear in these animal models, it is possible that the underexcitability in DEND patients is severe enough to result in a similar loss of β-cell mass.

ATP-binding mutations in KCNJ11 underlie a NDM phenotype. Several mutations of the putative ATP-binding site (R50P, Y330C, and F331I) have been reported in association with NDM (32,37). Generally, these cause milder nonsyndromic NDM, but R50P, like G334D, is also associated with DEND (36). Similar to G334D, R50P abolishes ATP sensitivity of recombinant channels without altering channel gating and increases sensitivity to stimulatory Mg nucleotides. Thus, it is becoming evident that the extent of channel overactivity, rather than the mechanism of overactivity (binding vs. gating), is a more reliable predictor of the severity of the disease. Given the similarities between G334D and R50P mutations, reflecting a common mechanism of action, one may expect future reports of DEND associated with ATP-binding site mutations that abolish ATP inhibition.

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