Original Article

ATP and Sulfonylurea Sensitivity of Mutant ATP-Sensitive K^+ Channels in Neonatal Diabetes

Implications for Pharmacogenomic Therapy

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The prediction that overactivity of the pancreatic ATP-sensitive K^+ channel (K_{ATP} channel) underlies reduced insulin secretion and causes a diabetic phenotype in humans has recently been borne out by genetic studies implicating "activating" mutations in the Kir6.2 subunit of K_{ATP} as causal in both permanent and transient neonatal diabetes. Here we characterize the channel properties of Kir6.2 mutations that underlie transient neonatal diabetes (I182V) or more severe forms of permanent neonatal diabetes (V59M, Q52R, and I296L). In all cases, the mutations result in a significant decrease in sensitivity to inhibitory ATP, which correlates with channel "overactivity" in intact cells. Mutations can be separated into those that directly affect ATP affinity (I182V) and those that stabilize the open conformation of the channel and indirectly reduce ATP sensitivity (V59M, Q52R, and I296L). With respect to the latter group, alterations in channel gating are also reflected in a functional "uncoupling" of sulfonylurea (SU) block: SU sensitivity of I182V is similar to that of wild-type mutants, but the SU sensitivity of all gating mutants is reduced, with the I296L mutant being resistant to block by tolbutamide (≤10 mmol/l). These results have important implications for the use of insulinotropic SU drugs as an alternative therapy to insulin injections. *Diabetes* 54:2645–2654, 2005

In the pancreatic β-cell, the ATP-sensitive K^+ channel (K_{ATP} channel) couples membrane excitability to insulin secretion (1). Increased glucose metabolism leads to elevated cytosolic ATP/ADP, closure of K_{ATP} channels at the plasma membrane, and membrane depolarization. The resulting activation of voltage-sensitive Ca^{2+} channels causes a rise in $[Ca^{2+}]_i$, which serves as the stimulus for insulin vesicle exocytosis. Sulfonylureas (SUs), which are major hypoglycemic agents used in treating type 2 diabetes, promote insulin secretion by specifically binding the regulatory SUR1 subunit of K_{ATP} and inhibiting K_{ATP} current (2), thus underscoring the central role of K_{ATP} in the regulation of insulin secretion.

Implicit in the paradigm of excitation-secretion coupling is that alterations in K_{ATP} currents should disrupt electrical signaling in the β-cell and, thereby, alter insulin release. Specifically, decreased metabolic flux, decreased sensitivity of K_{ATP} to inhibitory ATP, or increased density of K_{ATP} channels should all lead to abnormally high K_{ATP} activity and relative hypoinsulinism. In such scenarios, a diabetic phenotype is predicted; indeed, a severe neonatal diabetic phenotype is observed in mice lacking the β-cell glucokinase gene (3,4) and transgenic mice expressing β-cell K_{ATP} channels with reduced sensitivity to inhibitory ATP (i.e., "overactive" K_{ATP}) (5). A series of genetic studies have recently demonstrated that heterozygous, missense mutations in the pore-forming Kir6.2 subunit of the K_{ATP} channel underlie neonatal diabetes in humans, accounting for both permanent and transient forms of the disease (6–11). Rare in its occurrence (1:400,000 births), neonatal diabetes is usually diagnosed within the first 3 months of life and requires insulin administration to treat the hyperglycemia (12). In the milder, transient neonatal diabetes (TND), hyperglycemia usually resolves within 18 months after the diagnosis, whereas the equally common permanent neonatal diabetes (PND) requires insulin treatment for life. In both PND and TND, β-cell dysfunction is likely the primary lesion. However, in TND, it is proposed that expansion of β-cell mass compensates for the β-cell defect and underlies the remission phase (13). In one important finding, the administration of SUs to several neonatal diabetic patients carrying Kir6.2 mutations was sufficient to treat the hyperglycemia, thereby implicating K_{ATP} "overactivity" as causal in the suppressed insulin release (6,14).

All neonatal diabetes–associated Kir6.2 mutations examined to date have been shown to decrease ATP sensitivity of reconstituted K_{ATP} channels (7,10,15). Some of the mutations are in putative ATP-binding residues (e.g., R50, I182, R201), but others lie well away from any predicted binding regions. These latter mutations are likely to reduce ATP sensitivity by stabilizing the open state relative to the closed state (16); in fact, this has been demonstrated

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K_{ATP} channel, ATP-sensitive K^+ channel; PIP_2, phosphatidylinositol 4,5-bisphosphate; PND, permanent neonatal diabetes; SU, sulfonylurea; TND, transient neonatal diabetes.

for some PND mutations (15). Little attention has been paid to the sensitivity of different mutations to insulinotropic SU drugs. This is an important issue given that other mutations that alter open-state stability have been shown to dramatically affect SU sensitivity (17,18) and that SU therapy holds the promise of an alternative to insulin treatment for neonatal diabetes patients (6,14).

In the present study, we characterized Kir6.2 mutations that arguably underlie the mildest form of neonatal diabetes, TND (I182V), as well as the more severe, permanent forms of the disease (V59M, Q52R, and I296L). In all cases, mutations led to decreased ATP sensitivity. The mutations can be mechanistically classified as those that appear to directly alter ATP binding (I182V) and those that indirectly alter ATP sensitivity through changes in gating behavior (V59M, Q52R, and I296L). With respect to the latter group, a concomitant decrease in the sensitivity of reconstituted K_{ATP} channels to two clinically prescribed SUs (tolbutamide and glibenclamide) was observed in intact cells and excised patches. These findings have potentially important implications for the treatment of diabetic patients carrying Kir6.2 mutations and suggest that SU dosing may need to be considered on a case-by-case basis.

RESEARCH DESIGN AND METHODS

Molecular biology. Kir6.2 was cloned into the *Eco*RI/*Cla* I site of pCMV6b, and the parental plasmid DNA was used to generate Kir6.2 mutations using the QuickChange Site Directed Mutagenesis kit (Stratagene, La Jolla, CA). SUR1 was cloned into the pECE expression vector. The nucleotide sequences of the mutant Kir6.2 constructs were verified by fluorescence-based cycle sequencing using AmpliTaq DNA polymerase FS (PerkinElmer, Foster City, CA) and an ABI Prism DNA sequencer (PerkinElmer).

Expression of K_{ATP} channels in COSm6 cells. COSm6 cells were plated at a density of $\sim 2.5 \times 10^5$ cells/well (30-mm, six-well dishes) and cultured in Dulbecco's modified Eagle's medium with 10 mmol/l glucose supplemented with FCS (10%). The following day, a transfection cocktail was prepared by adding 6 μl of FuGENE 6 Transfection Reagent (Roche, Indianapolis, IN), 600 ng each of pCMV6b-Kir6.2 and pECE−green fluorescent protein plasmid, and 1 μg pECE-SUR1 (per well). After a 45-min incubation at room temperature, the cocktail was added directly to the growth medium on the plated cells. Cells were assayed for K_{ATP} currents by patch-clamp measurements 2–4 days after transfection.

Rubidium flux experiments. ^{86}RbCl (1 mCi/ml) was added in fresh growth medium 48 h after transfection, and cells were incubated for an additional 24 h before Rb^+ flux was assayed. For efflux measurements, cells were preincubated for 2 min at 25°C in Krebs-Ringer solution with or without metabolic inhibitors (2.5 μg/ml oligomycin plus 1 mmol/l 2-deoxy-D-glucose [Sigma, St. Louis, MO]). At selected time points, the solution was aspirated from the wells and replaced with fresh solution. The $^{86}Rb^+$ in the aspirated solution was counted in a scintillation solution. Rb^+ efflux curves were fit by assuming that a single non-K_{ATP} conductance was active in untransfected cells, determined as

$$\text{Rb efflux} = \exp(-k_1 \cdot t) \quad (1)$$

where exp is an exponential function, t is time, and k_1 is the rate constant for efflux through the endogenous, non-K_{ATP} pathway.

In transfected cells, efflux curves were fit by assuming that both a K_{ATP} and a non-K_{ATP} conductance pathway were active in parallel and were determined as

$$\text{Rb efflux} = \exp[(-k_1 + k_2) \cdot t] \quad (2)$$

where k_1 is the rate constant for efflux through the endogenous, non-K_{ATP} pathway (obtained from untransfected cells by Eq. 1) and k_2 is the rate constant for efflux through the K_{ATP} pathway. k_2 is then directly proportional to the K_{ATP} conductance.

Patch-clamp measurements. Patch-clamp studies were performed at room temperature in an oil-gate chamber that allowed the solution bathing the exposed surface of the isolated patch to be changed rapidly. The standard bath (intracellular) and pipette (extracellular) solution (K-INT) used in these experiments was comprised of 140 mmol/l KCl, 10 mmol/l K-HEPES, and 1 mmol/l K-EGTA (pH 7.3). Microelectrode resistance was typically 0.5–1 MΩ when filled with K-INT solution. Membrane patches were voltage clamped with an Axopatch 1B patch-clamp amplifier (Axon, Foster City, CA). Phosphatidylinositol 4,5-bisphosphate (PIP_2; 5 μg/ml in K-INT) was bath sonicated in ice for 30 min before being used. Glibenclamide was dissolved as a 1 mol/l stock solution in DMSO and diluted to <1% DMSO in K-INT. Tolbutamide was prepared as a 100 mmol/l stock solution in 150 mmol/l KOH and diluted to the appropriate working concentration in K-INT (pH 7.3). PIP_2 was obtained from Boehringer Mannheim. Tolbutamide, glibenclamide, and ATP were purchased from Sigma. All currents were measured at a membrane potential of −50 mV (pipette voltage = +50 mV). Data were normally filtered at 0.5–3 kHz, and signals were digitized at 22 kHz (Neurocorder; Neurodata, New York, NY). Experiments were digitized into a microcomputer using Clampex 8.2 software (Axon), and off-line analysis was performed using Clampfit 8.2 (Axon) and Microsoft Excel programs. Wherever possible, data are presented as means ± SE. Microsoft Solver was used to fit data by a least-squares algorithm.

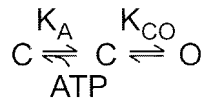

FIG. 1. A three-state model of K_{ATP} channel activity.

Estimation of $P_{o,zero}$. Two approaches, one indirect and one direct, were used to estimate the open probability in zero ATP ($P_{o,zero}$) after isolated membrane patches containing multiple channels were excised (19).

PIP_2 method (indirect). PIP_2 was added to the patch until the current reached a saturating level (I_{PIP2}). This was assumed to represent a maximum $P_{o,zero}$ of ~0.9 (20). The fractional change in current was calculated as

$$\text{Fractional change} = I_{initial}/I_{PIP2} \quad (3)$$

where $I_{initial}$ is the initial current. $P_{o,zero}$ was then estimated as

$$P_{o,zero} = 0.9/\text{fractional change} \quad (4)$$

Noise analysis method (direct). In addition, the mean $P_{o,zero}$ was estimated from stationary fluctuation analysis of macroscopic currents (21,22) on short (<1 s) recordings of currents in zero or 5 mmol/l ATP or 10 mmol/l spermine (for estimation of ATP-independent noise). Currents (corresponding to ~1–1000 channels) were filtered at 1 kHz and digitized at 3 kHz with 12-bit resolution. The mean patch current (I) and variance (σ) (2) in the absence of ATP were obtained by subtracting the mean current and variance in 5 mmol/l ATP or 10 mmol/l spermine (i.e., assuming all channels were fully closed or blocked). The single channel current (i) was assumed to be −3.75 pA at −50 mV, corresponding to a wild-type single channel conductance of 75 pS (23). $P_{o,zero}$ was then estimated as

$$P_{o,zero} = 1 - [\sigma^2/(i \cdot I)] \quad (5)$$

Three-state model for K_{ATP} channel activity. Detailed kinetic analysis of K_{ATP} channel activity predicts complex models, but a simplified three-state model (19,20) can account qualitatively and quantitatively for the essential features of macroscopic K_{ATP} channel activity. Accordingly, we examined whether this simple model (Fig. 1), in accounting for the effects of mutations on gating behavior and nucleotide sensitivity, could be used predictively to account for observed changes in drug sensitivity.

The ATP binding affinity (K_A) and open-close state equilibrium (K_{CO}) for wild-type channels were assumed to be 3 μmol/l and 0.60, respectively. To obtain predictions of behavior for mutant channels (as in Fig. 8), only one or the other parameter was changed. For I182V, it was necessary to change K_A (increased by sixfold) without changing K_{CO}. For Q52R, V59M, and I296L channels, it was necessary to change K_{CO} (increased by 40-, 6-, and 600-fold, respectively) without changing K_A.

Dose-response curves for SU (glibenclamide and tolbutamide) inhibition were described by the sum of two Hill equations (reflecting two components of inhibition):

$$G_{rel} = f/[1 + (SU/K_1)] + (1 - f)/[1 + (SU/K_2)], \quad (6)$$

where G_{rel} is the relative conductance, f is the fraction of high-affinity block, and K_1 and K_2 are the half-maximal concentration of high- and low-affinity block, respectively.

It is clear that the relative magnitude of the two components was changed by the mutations as was the apparent affinities. We postulated, based on previous findings (17,18), that these changes resulted directly from changes in open-state stability. As shown in Fig. 8, to model the effects of different mutations on SU sensitivity, it was necessary to assume only that the fraction

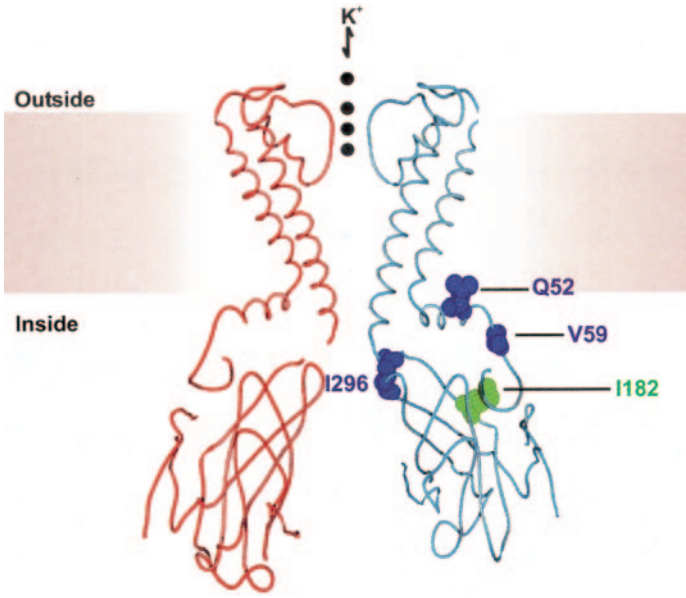

FIG. 2. Kir6.2 mutations from patients with PND and TND. Ribbon diagram of two of the four Kir6.2 subunits that form the K^+-selective pore in K_{ATP}. Kir6.2 structure is based on the crystal structure of KirBac1.1. Shown are several Kir6.2 residues mutated in syndromic PND (Q52, V59, I296), and TND (I182V). Residues are classified as those involved directly in ATP binding (green) or channel gating (blue).

of high-affinity block (f) and the half-maximal blocking concentrations (K_1 and K_2) of the high- and low-affinity components were each dependent on the open-state stability (defined by K_{CO}), according to the following three formulas:

$$f = 1/[1 + (K_{CO}^{0.5})/x] \quad (7)$$

where $x = 20$ or 1 for glibenclamide (Fig. 2*C*) or tolbutamide (Fig. 2*D*) inhibition curves, respectively.

$$K_1 = K_{1(0)} \cdot K_{CO} \quad (8)$$

where $K_{1(0)}$ is equal to 1 μmol/l or 10 nmol/l for glibenclamide or tolbutamide inhibition, respectively.

$$K_2 = K_{2(0)} \cdot K_{CO}^3 \quad (9)$$

where $K_{2(0)}$ is much greater than 10 or 3 mmol/l for glibenclamide or tolbutamide inhibition, respectively.

RESULTS

Variable on-cell activity correlates with variable ATP sensitivity. To better understand the spectrum of K_{ATP}-induced neonatal diabetes and its underlying mechanisms, we chose to characterize the TND-causing I182V mutation and the three mutations implicated in more severe PND (Q52R, V59M, I296L) (Fig. 2). Mutant Kir6.2 subunits were coexpressed with the wild-type SUR1 subunit, and channel activity in intact cells was first screened by ^{86}Rb flux experiments. As shown in Fig. 3, $^{86}Rb^+$ efflux from cells transfected with wild-type K_{ATP} channels (Kir6.2 + SUR1) was low under basal conditions and was significantly activated by metabolic inhibition with oligomycin and 2-deoxyglucose to lower cellular ATP/ADP. In contrast, Rb^+ efflux from cells transfected with mutant Kir6.2 subunits was high under basal conditions and showed very little increase, if any, with metabolic inhibition. To obtain quantitative estimates of conductances, a simple two-pathway (K_{ATP} + non-K_{ATP}) model for Rb^+ efflux was assumed (see RESEARCH DESIGN AND METHODS). Metabolic inhibition reduced the Rb^+ efflux rate in untransfected cells; Rb^+ efflux time courses in basal and metabolically inhibited conditions for untransfected cells were thus fit by Eq. 1, which assumes a single non-K_{ATP} conductance is active in each condition. The rate constants thus obtained were used to estimate rate constants for K_{ATP} conductance under basal conditions and metabolic inhibition in transfected cells using Eq. 2. These rate constants were then assumed to be directly proportional to K_{ATP} conductance. Figure 4*A* shows the estimated K_{ATP} conductance under basal and metabolically inhibited conditions for each transfected construct. Maximal activated conductance was similar for all constructs except I296L. This mutation gave consistently lower fluxes, a point considered below (see DISCUSSION), but the basal flux was almost maximal.

We next examined K_{ATP} channel activity directly in voltage-clamped membrane patches from transfected cells. For each patch, we estimated the current in basal physiological conditions (on-cell current) and the measured maximal K_{ATP} current after patch excision (data not shown). In Fig. 4*B*, relative K_{ATP} current activation on-cell from patch-clamp analysis is plotted against the relative K_{ATP} activation in intact cells estimated by Rb^+ efflux for each mutation. Both measures give correlated activities, suggesting that each method reflects the degree of overactivity in intact cells. To examine the correlation between increased K_{ATP} activity in intact cells and ATP sensitivity, the latter was measured directly in excised membrane patches (Fig. 5). All mutant K_{ATP} channels exhibited a significant decrease in ATP inhibition, ranging from twofold (V59M) to >100-fold (I296L) (Table 1). Channel overactivity in intact cells correlated reasonably well with reduction in ATP sensitivity, but a direct correlation between ATP insensitivity and severity of the neonatal diabetes was not absolute, as the TND-causing I182V mutation was approximately twofold less sensitive than V59M, a mutation that is associated with severe, syndromic PND (see DISCUSSION). It is conceivable that channel trafficking was altered by neonatal diabetes mutations so that a higher current may partly reflect higher expression levels. As shown in Table 1, patch current density and calculated channel density in this recombinant system were not significantly different between wild-type and mutant channels.

Mutations may affect ATP sensitivity by reduced ATP binding or indirectly by changes in the relative stability of the open state. Mutations could mechanistically reduce ATP sensitivity directly by reducing ATP affinity or indirectly by affecting the intrinsic opening ability. Previous studies have characterized mutations throughout Kir6.2 that stabilize the open state of the channel relative to the closed state, even in the absence of ATP, and thereby decrease the apparent ATP sensitivity (19,20,24,25). To elucidate potential mechanisms in neonatal diabetes mutations, we measured the open probability in the absence of ATP ($P_{o,zero}$), using two independent methods: PIP_2 activation (19) and nonstationary noise analysis (22) (see RESEARCH DESIGN AND METHODS). Each method gave very similar results (see Fig. 8*A*). $P_{o,zero}$ of wild-type channels was ~0.4 and was not altered by the TND-associated I182V mutation (which reduced ATP sensitivity approximately

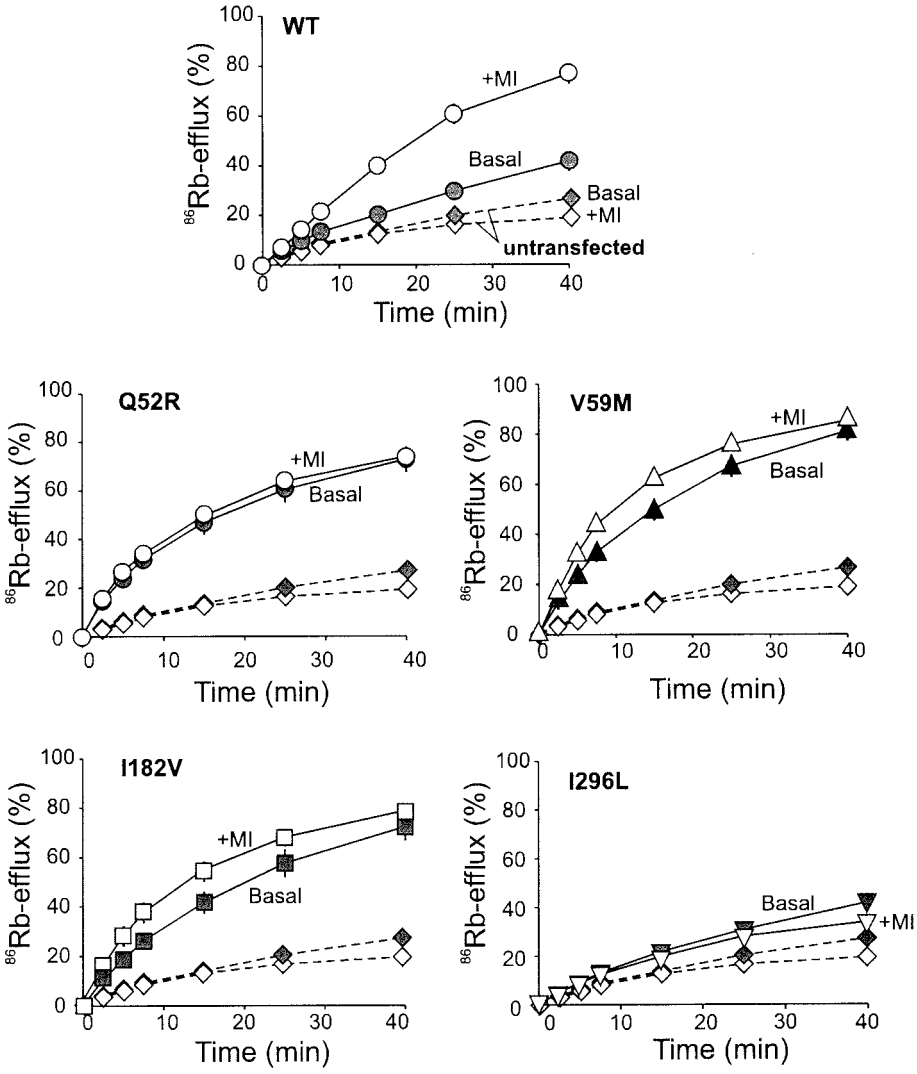

FIG. 3. $^{86}Rb^+$ efflux from untransfected COSm6 cells and cells expressing wild-type (WT) or mutant K_{ATP}. Data are the percent of efflux of $^{86}Rb^+$ as a function of time in basal conditions or in the presence of metabolic inhibition (MI; see RESEARCH DESIGN AND METHODS) for reconstituted WT and mutant K_{ATP} channels (Kir6.2 + SUR1). All transfections were done in parallel. Graphs show compiled data (means ± SE) from three to eight experiments.

fivefold), consistent with the mutation directly affecting ATP binding. However, each of the PND-associated mutations caused a significant increase in $P_{o,zero}$ (Table 1), indicative of an indirect effect on ATP sensitivity. This differential mechanistic basis for channel activation could have profound consequences, both for the severity of the resultant neonatal diabetes and for the possibilities for SU therapy (see DISCUSSION).

SU sensitivity is reduced in parallel with increases in open-state stability. Previously, it has been shown that increasing the open-state stability of K_{ATP}, either through mutagenesis or the application of PIP_2, functionally "uncouples" the regulatory SUR1 subunit from the ion-conducting Kir6.2 subunit (17,18). This uncoupling is reflected as a reduction or loss of high-affinity SU inhibition through the SUR1 subunit. For Kir6.2 mutations that increase the open-state stability, we may therefore expect to find a concomitant decrease of high-affinity SU block in addition to a decrease in apparent ATP affinity. To test this possibility, we examined SU sensitivity, in intact cells using $^{86}Rb^+$ flux measurements and in inside-out patch-clamp experiments.

As shown in Fig. 6, glibenclamide almost completely inhibited Rb^+ efflux through wild-type channels at clinically effective doses (i.e., in the nanomolar range). It has previously been demonstrated that SU inhibition in excised membrane patches is biphasic, with high- and low-affinity components (17,26). Although a biphasic sensitivity has not been previously demonstrated in intact tissue, it is evident in the SU sensitivity of mutant K_{ATP} channels in intact COS cells (Fig. 6), where Rb^+ efflux is inhibited to a plateau level by micromolar glibenclamide. For mutants that exhibit increased open-state stability, the high-affinity component decreases (Q52R, V59M, I296L) and the affinity itself decreases (Q52R, I296L). The highest open-state stability mutant (I296L) is almost completely insensitive to glibenclamide in the nanomolar range, but a reduced high-affinity component and lower affinity is also quite clear for the Q52R mutant.

Glibenclamide is a hydrophobic drug (27), and inhibition of K_{ATP} channels in excised patches was largely irreversible over the experimental period. However, tolbutamide, a first generation SU, is more hydrophilic, can be rapidly reversed, and has been a useful model SU in excised membrane patch experiments. As shown in Fig. 7, wild-type channels showed typical biphasic block by tolbutamide in inside-out membrane patches (17,26), and sensitivity was unaltered in the I182V mutation (Fig. 7*B*). This finding is again consistent with the interpretation that the I182V substitution directly alters ATP binding/trans-

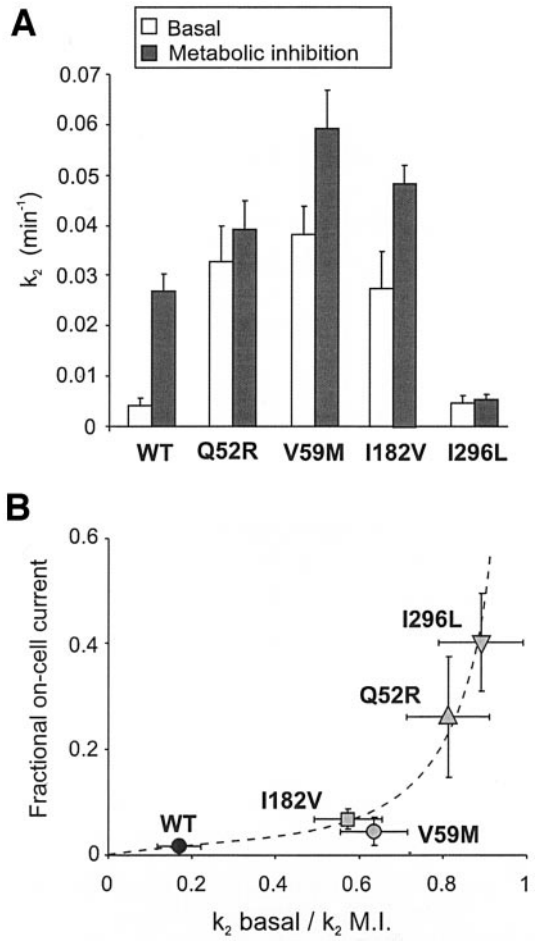

FIG. 4. Increased activity of mutant K_{ATP} channels in intact COSm6 cells. *A*: Rb^+ efflux rate constants for K_{ATP}-dependent pathway (k_2) for wild-type (WT) or mutant K_{ATP} channels under basal conditions and metabolic inhibition (MI; see RESEARCH DESIGN AND METHODS). Data are means ± SE from three to eight experiments. (*B*) Fractional on-cell current in membrane patches versus ratio of k_2 in basal conditions to k_2 in MI from Rb^+ efflux experiments (as in Fig. 3).

duction but does not affect gating (28), and thus the mutant channels remain functionally "coupled" to SUR1.

In sharp contrast, functional uncoupling from the SUR1 subunit is again reflected by a decrease in high-affinity fractional SU block in each of the mutations that increase the open-state stability (Q52R, V59M, I296L) (Table 1). It is interesting that I296L channels, which show the highest open-state stability, are completely insensitive to inhibition by tolbutamide at concentrations as high as 10 mmol/l. However, when wild-type Kir6.2 and I296L subunits were coexpressed to generate heterozygous channels, the SU sensitivity was partially restored, but with a significant decrease in the high-affinity component of block (17% high-affinity tolbutamide block for heterozygous I296L:wild-type channels vs. 55% block for homomeric wild-type channels) (Fig. 7*B*).

DISCUSSION

Reduced ATP sensitivity underlies both TND and PND. It is now clear that mutations in the Kir6.2 subunit of K_{ATP} represent a common cause of neonatal diabetes in humans (6–11). To better understand the mechanism(s) by which these mutations alter excitation-secretion coupling and their effect on SU sensitivity, we chose to characterize Kir6.2 mutations implicated in the mildest, transient form of the disease (I182V) (10) and those associated with more severe PND (Q52R, V59M, I296L) (6–9). In all cases, the sensitivity of homomeric mutant K_{ATP} to inhibitory ATP was reduced relative to wild-type K_{ATP}, with the following order of sensitivity: WT > V59M > I182V > Q52R > I296L. The syndromic PND-linked V59M mutation exhibited comparable if not greater sensitivity to ATP than did the TND-associated I182V ($K_{1/2ATP}$ = 15 [V59M] and 31 [I182V] μmol/l), reflected in a similar degree of "overactivity" in intact cells (Fig. 4*B*). Assuming the behavior of reconstituted channels reflects K_{ATP} activity in the β-cell, these data do not demonstrate an absolute correlation between the severity of the diabetes (TND, PND, and syndromic PND) and the degree of ATP sensitivity. Such a positive correlation has been previously suggested based on a comparison of the less severe functional defects of a PND-associated mutation (R201C) with syndromic PND-associated mutations (Q52R, V59G) (15). Taken together, these data suggest a complex phenotype in which the primary defect is at the level of the β-cell and altered insulin secretion, but they also indicate that additional genetic and/or environmental factors may modulate the severity of the disease (TND, PND, and syndromic PND). It is interesting that K_{ATP} in skeletal muscle (Kir6.2 + SUR2A) is implicated in peripheral insulin sensitivity based on the observation that both Kir6.2- and SUR2A-null mice exhibit increased glucose uptake in skeletal muscle and adipose tissue (29,30). The mouse models, therefore, predict that "overactive" K_{ATP}, as observed in neonatal diabetes, may suppress glucose uptake and contribute to the hyperglycemia. Whether neonatal diabetes patients, in addition to having suppressed insulin release, also exhibit insulin insensitivity remains to be determined.

An important distinction between the present study and that of Proks et al. (15) is that the order of sensitivity in Proks et al.'s report was based on the ATP sensitivity of heteromeric channels (generated by the expression of channels from equal amounts of wild-type Kir6.2 and mutant Kir6.2 cRNA), whereas our analysis is of homomeric channels. We have found that in similar experiments with mixed expression of wild-type and mutant Kir6.2 subunits, the rank order of mutant ATP sensitivity is unaltered (data not shown). However, a quantitative analysis of mixed expression is very complicated, given the fact that six distinct combinations of wild-type and mutant subunits will generate six different channel types, each with its own ATP sensitivity. Whether mutant alleles of Kir6.2 will be expressed equally in vivo and in different tissues is also not known. Given these complexities, the analysis of homomeric channels may be the most straightforward approach to understanding mutational effects on channel properties.

Finally, the I296L mutant channel consistently exhibited a lower activity in Rb^+ efflux experiments, despite a channel density that was similar to that of wild-type channels in excised patches. This finding may reflect a lower transfection efficiency. However, because the I296L mutation resulted in the highest on-cell activity of all the neonatal diabetes mutants, it is also possible that this

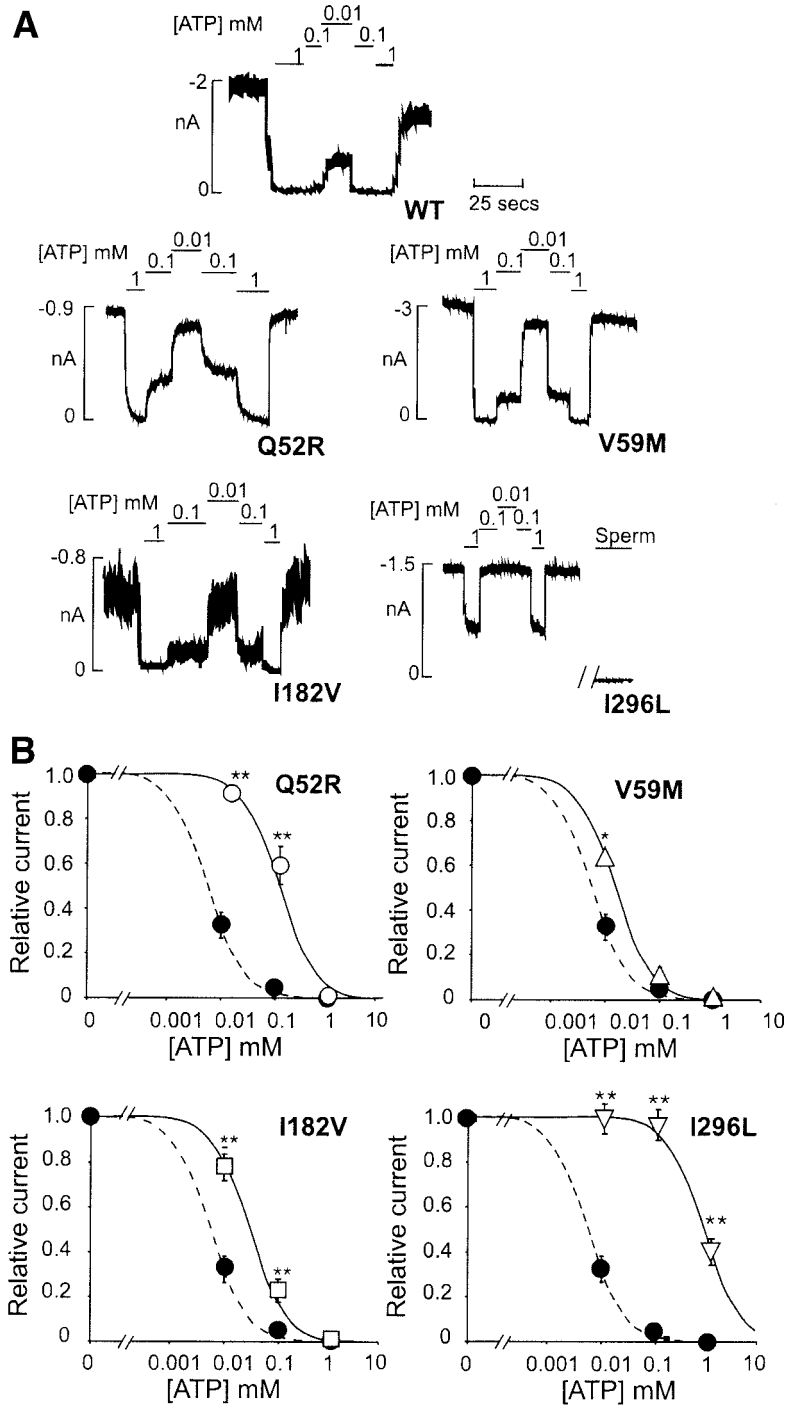

FIG. 5. ATP sensitivity of K_{ATP} currents from COSm6 cells coexpressing wild-type (WT) or mutant Kir6.2 and SUR1. *A*: Representative currents recorded from inside-out membrane patches containing WT or mutant K_{ATP} channels at −50 mV in K-INT solution (see RESEARCH DESIGN AND METHODS). Patches were exposed to different ATP concentrations, and baseline current was determined by the addition of ATP (10 mmol/l) or spermine (10 mmol/l). *B*: Steady-state dependence of membrane current on ATP (relative to current in zero ATP [I_{rel}]) for WT and mutant channels. Data points represent means ± SE (n = 5–15 patches). The fitted lines correspond to least squares fits of the Hill equation (relative current = $100/(1 + [ATP/K_i]^H)$, with H fixed at 1.3 (see Table 1). Closed symbols and dashed lines represent data and fits for WT channels. $P < 0.05$ and $^{**}P < 0.01$ vs. WT K_{ATP} by unpaired Student's t test.

elevated K^+ conductance was toxic to the cells, so that we were experimentally selecting for a subpopulation of cells that are resistant to elevated K^+ conductance in excised patch experiments.

Differential SU sensitivity of neonatal diabetes mutations. SUs block pancreatic K_{ATP} currents and stimulate insulin release. The high-affinity component of block by glibenclamide (IC_{50} ~1 nmol/l) or tolbutamide (IC_{50} ~1 μmol/l) results from binding to the SUR1 subunit (26), whereas the Kir6.2 subunit underlies the low-affinity component (IC_{50} ~1,000 × high affinity). A direct correlation between gating behavior of the channel and SU sensitivity has been previously demonstrated (17,18). The consequence of such a relation is that mutations in Kir6.2 that increase the stability of the open state not only reduce the apparent ATP sensitivity, but concomitantly decrease the high-affinity SU block. This is interpreted as a functional "uncoupling" of the regulatory SUR1 subunit from the Kir6.2 channel (17).

We thus predict that PND mutations that increase open-state stability (V59M, Q52R, and I296L) should also reduce SU sensitivity. This hypothesis is consistent with the previous observation that the block of whole-cell K^+ currents was not complete in 500 μmol/l tolbutamide for PND mutant K_{ATP} (15). Indeed, the gating mutations Q52R and V59M but not the ATP-binding mutant I182V exhibited

TABLE 1
Isolated membrane patch properties of neonatal diabetes–associated K_{ATP} channels

Mutation	$K_{i,ATP}$ (μmol/l [n])*	$P_{o,max}$ (n)†	Channel density	Tolbutamide sensitivity* High-affinity IC_{50} (mmol/l)	High-affinity f	Low-affinity IC_{50} (mmol/l)
Wild type	5.8 (15)	0.35 ± 0.05 (12)	359 ± 105	0.001 (12)	0.55	2.0 (12)
I182V	31.2 (11)	0.42 ± 0.05 (13)	147 ± 63	0.001 (20)	0.6	2.3 (20)
V59M	15.6 (10)	0.91 ± 0.01 (12)	826 ± 213	0.001 (9)	0.2	2.5 (9)
Q52R	125.2 (7)	0.93 ± 0.02 (10)	361 ± 121	0.026 (12)	0.35	12 (12)
I296L	770.8 (5)	0.95 ± 0.02 (9)	265 ± 150	ND	—	ND
I296L:wild type	39.3 (7)	ND	ND	0.001 (6)	0.17	10 (6)

Data are means ± SE. *Analysis was performed on compiled data of n values. †Estimated from nonstationary noise analysis (direct method). Channel density was calculated as $I/(P_{o,max} \times i)$ from maximum patch current in zero ATP (I) and assuming a single channel current (i) = 3.75 A at −50 mV. No values were significantly different from wild type ($P = 0.06–0.9$). f, fraction of high-affinity block; ND, not determined.

a decrease in the fractional block by the high-affinity component. The Q52R and I296L mutants also exhibited a significant decrease in the sensitivity of the low-affinity block, with I296L mutant channels being completely refractory to inhibition by tolbutamide ≤10 mmol/l. This implies that in addition to a reduced SU sensing through SUR1, the I296L and Q52R mutations also reduce SU action through the low-affinity site on Kir6.2, either directly or allosterically.

A mechanistic model for nucleotide and SU sensitivity. Diabetes-causing Kir6.2 mutations can be classified mechanistically as those that directly alter ATP binding and/or transduction of the ATP-mediated signal (I182V) versus those that indirectly affect ATP affinity by increasing the open-state stability (Q52R, V59M, I296L). The data are consistent with recent characterizations of other PND mutations that affect ATP binding (R201C, R201H, I182V) or alter gating behavior, leading to a secondary decrease in ATP sensitivity (V59G, Q52R) (10,15).

Models of K_{ATP} channel gating and nucleotide and SU sensitivity have been developed. To have predictive use, such models should account for channel gating (in the absence of ATP), nucleotide sensitivity, and drug sensitivity, and by adjusting relevant parameters, should be able to account for the effects of mutations on channel function. The most complex models (20,28,31) account for the tetrameric nature of the channel and could potentially account for heteromeric channels consisting of wild-type and mutant subunits. However, a relatively simple three-state model (20) (see RESEARCH DESIGN AND METHODS, Fig. 1) can account for all of the pertinent features of macroscopic channel activity and has predictive qualities for the interpretation of nucleotide and drug sensitivities of neonatal diabetes mutants. We therefore postulated that mutational effects on SU sensitivity result directly from changes in open-state stability (17,18).

As shown in Fig. 8, the simplified three-state model quantitatively predicted the effects of neonatal diabetes mutations on each of the three features, $P_{o,zero}$, ATP sensitivity, and SU sensitivity, by adjusting only two parameters, K_A and K_{CO}, which are the equilibrium constants that describe ATP affinity and open-state stability, respectively. By assuming that both the affinity and the fraction of high-affinity SU block are dependent on only K_{CO}, the steady-state SU sensitivities of wild-type and mutant channels can be predicted. As discussed below, this analytical approach may be a useful predictor of SU efficacy in human therapy trials.

Implications for SU therapy of neonatal diabetes. Recently, SUs have been shown to be effective, at least in the short-term, in maintaining control of blood glucose levels in several PND patients in which Kir6.2 mutations are causal (F331I, V59M, and R201H) (6,14). Given that blood glucose levels and HbA_{1c} values were stable after discontinuation of insulin in these patients, it has been proposed that oral SUs can be an alternative therapy to subcutaneous insulin injections in patients with PND because of Kir6.2-activating mutations. The present results indicate, however, that the efficacy of SU therapy is likely to depend on the nature of the Kir6.2 mutation. Assuming that the SU sensitivity of reconstituted channels reflects the sensitivity of channels in vivo, carriers of the diabetes-causing I296L and similarly behaving Kir6.2 mutations may be less responsive to SU therapy. In one interesting case,

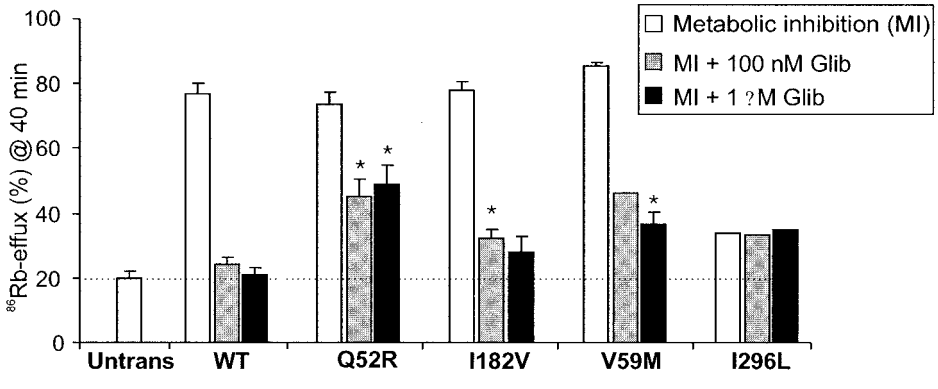

FIG. 6. Glibenclamide inhibition of $^{86}Rb^+$ efflux from cells expressing wild-type (WT) or mutant K_{ATP}. Data are the percent of efflux of $^{86}Rb^+$ at 40 min in the presence of metabolic inhibition (see RESEARCH DESIGN AND METHODS) for WT and mutant Kir6.2 + SUR1. Data are means ± SE, n = 3–8). Glibenclamide (Glib) was included at the indicated concentrations. The dashed line indicates efflux from untransfected cells. *$P < 0.05$ vs. WT K_{ATP} by unpaired Student's t test.

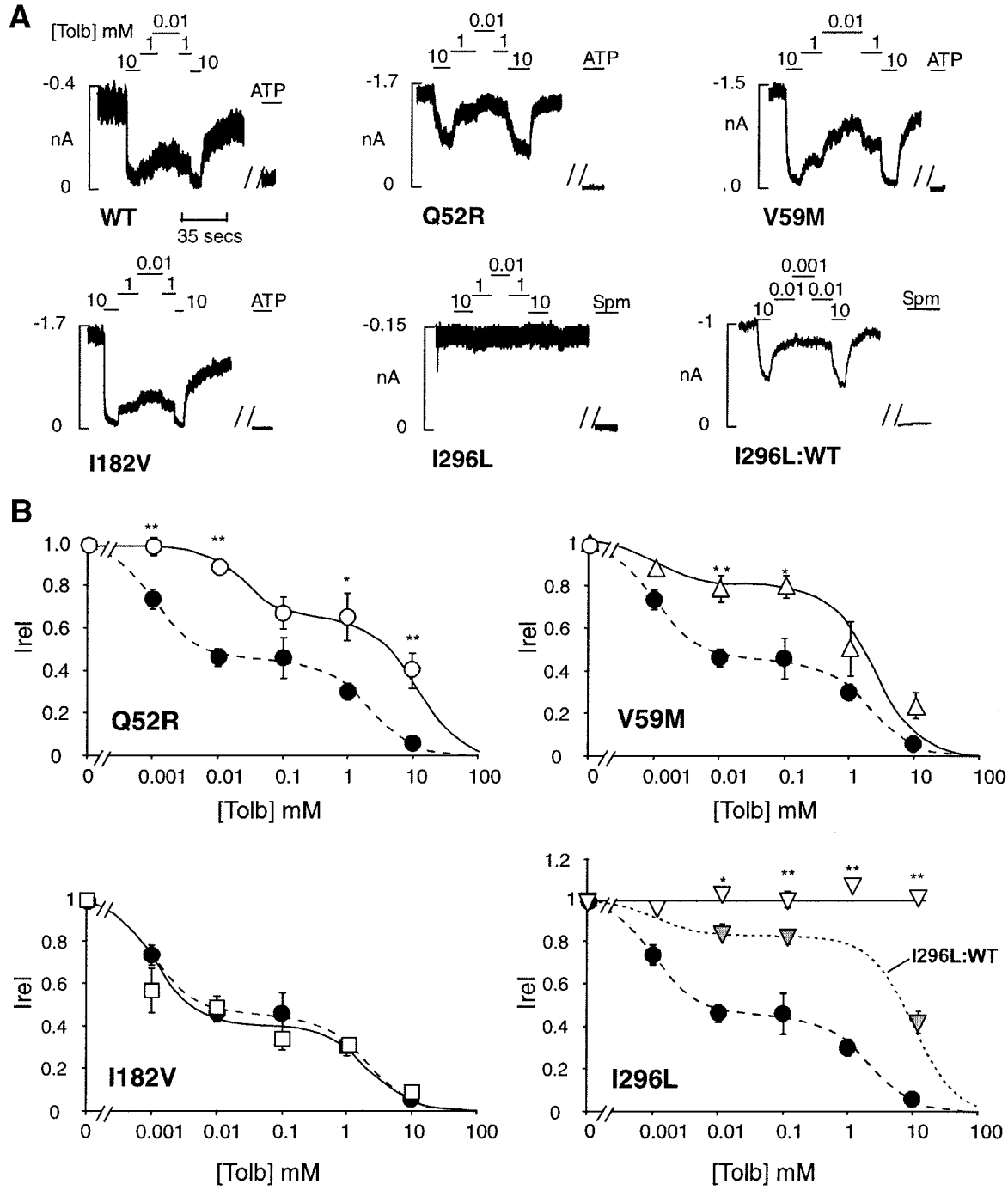

FIG. 7. Tolbutamide sensitivity of K_{ATP} currents from COSm6 cells coexpressing wild-type (WT) or mutant channels. *A*: Representative currents recorded from inside-out membrane patches containing WT or mutant K_{ATP} channels (Kir6.2 + SUR1) at −50 mV. Patches were exposed to differing concentrations of tolbutamide (Tolb), as shown. For all mutant channels, the baseline was determined by the application of spermine (10 mmol/l) or ATP (10 mmol/l). *B*: Steady-state dependence of membrane current on Tolb (relative to current in zero Tolb [I_{rel}]) for WT and mutant channels (from records such as those shown in *A*). Data points represent means ± SE (n = 9–20 patches). For all channels, the lines are fits of the sum of two Hill components (26), both being of the form $I_{rel} = 1/[1 + (Tolb/IC_{50})^H]$, with H fixed at 1.3 in each case. The relative fraction and IC_{50} values of each component were varied (see Table 1). *$P < 0.05$ and **$P < 0.01$ vs. WT K_{ATP} by unpaired Student's t test.

a PND patient with the syndromic V59M mutation was responsive to SU therapy and discontinued insulin injections (6), thereby suggesting that despite a significant decrease in sensitivity of homomeric V59M mutant channels to SU inhibition, sufficient high-affinity inhibition was still able to induce insulin release. In the original report by Gloyn et al. (7), it was observed that a parent of the R201H proband was diagnosed as a baby with diabetes and had been effectively treated with tolbutamide for 46 years. The mutation R201H causes a significant loss of ATP sensitivity (7), but it is predicted to be an ATP-binding site mutation (16,32,33) and thus may not show loss of SU sensitivity.

Functional characterization of additional PND-associated mutations in Kir6.2, as well as additional clinical studies, will provide essential data regarding the efficacy

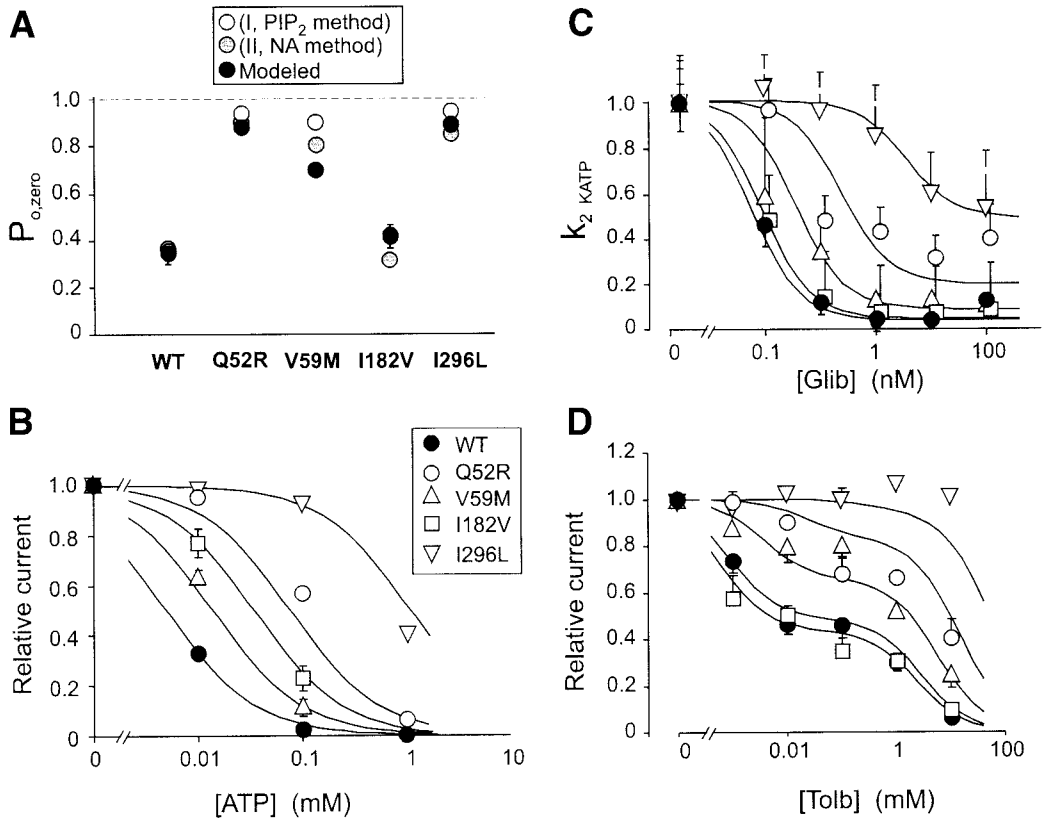

FIG. 8. Prediction of K_{ATP} channel properties by three-state model. *A*: $P_{o,zero}$ was estimated by both a direct (noise analysis) and an indirect (PIP_2) method and predicted by the given model parameters (see RESEARCH DESIGN AND METHODS). Data are means ± SE. *B*: ATP inhibition data for each mutant (taken from Fig. 5) together with predicted curves. *C*: Glibenclamide (Glib) dependence of ^{86}Rb efflux rate constant (K_2) for each mutant (from data in Fig. 6) together with model predictions. *D*: Tolbutamide (Tolb) dependence of channel activity for each mutant (from data in Fig. 7), together with predictions of the model. WT, wild type.

of SU therapy in treating K_{ATP}-induced diabetes and the possibility of tailoring individual therapy based on the underlying Kir6.2 mutation. The present results suggest that careful attention should be paid to the degree to which neonatal diabetes mutations cause a loss of ATP sensitivity by an increase in open-state stability. The greater the mutational effect on open-state stability is, the higher the SU dose necessary to achieve a given degree of channel inhibition will be. Thus, to achieve the same therapeutic effect (i.e., to obtain sufficient closure of K_{ATP} under appropriate physiological conditions), proportionally higher doses of an SU may be clinically required.

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