Original Article

Kir6.2 Mutations Associated With Neonatal Diabetes Reduce Expression of ATP-Sensitive K^+ channels

Implications in Disease Mechanism and Sulfonylurea Therapy

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Heterozygous missense mutations in the pore-forming subunit Kir6.2 of ATP-sensitive K^+ channels (K_{ATP} channels) have recently been shown to cause permanent neonatal diabetes mellitus (PNDM). Functional studies demonstrated that PNDM mutations reduce K_{ATP} channel sensitivity to ATP inhibition, resulting in gain of channel function. However, the impact of these mutations on channel expression has not been examined. Here, we show that PNDM mutations, including Q52R, V59G, V59M, R201C, R201H, and I296L, not only reduce channel ATP sensitivity but also impair channel expression at the cell surface to varying degrees. By tagging the PNDM Kir6.2 mutant V59G or R201H with an additional mutation, N160D, that confers voltage-dependent polyamine block of K_{ATP} channels, we demonstrate that in simulated heterozygous state, all surface channels are either wild-type or heteromeric channels containing both wild-type and mutant Kir6.2 subunits. Comparison of the various PNDM mutations in their effects on channel nucleotide sensitivity and expression, as well as disease phenotype, suggests that both channel-gating defect and expression level may play a role in determining disease severity. Interestingly, sulfonylureas significantly increase surface expression of certain PNDM mutants, suggesting that the efficacy of sulfonylurea therapy may be compromised by the effect of these drugs on channel expression. *Diabetes* 55:1738–1746, 2006

Pancreatic ATP-sensitive K^+ channels (K_{ATP} channels), each consisting of four pore-forming Kir6.2 subunits and four regulatory sulfonylurea receptor one (SUR1) subunits, link β-cell metabolism to insulin secretion (1–3). The activity of K_{ATP} channels is governed mainly by the dynamics of intracellular adenine nucleotides ATP and ADP at the channel site during glucose metabolism (1,4). Both nucleotides can stimulate or inhibit channel activity depending on their relative concentrations and whether Mg^{2+} is present. Inhibition of channels by nucleotides is mediated by the Kir6.2 subunit and does not require Mg^{2+} (5,6), whereas nucleotide stimulation is conferred by the SUR1 subunit and requires Mg^{2+} (7,8). The physiological activity of K_{ATP} channels in β-cells is thus a balance between nucleotide inhibition and nucleotide stimulation. During glucose stimulation, ATP concentrations increase and ADP concentrations decrease, resulting in K_{ATP} channel closure. Because K_{ATP} channels carry the dominant conductance in high-input resistance β-cells at resting state, closure of K_{ATP} channels leads to membrane depolarization, which in turn leads to opening of voltage-gated calcium channels, calcium influx, and insulin release.

Recent studies have established heterozygous missense mutations in Kir6.2 as a major cause underlying permanent neonatal diabetes mellitus (PNDM) (9–16). As Kir6.2 is also a constituent of K_{ATP} channel subtypes expressed outside of pancreas, including cardiac muscle, skeletal muscle, and brain, some mutations have been reported to cause muscle weakness, dysmorphic features, developmental delay, epilepsy, in addition to neonatal diabetes (referred to as DEND syndrome) (15,17). Functional studies of mutant channels have revealed reduced ATP sensitivity as a common defect (11,18,19). While reduced channel sensitivity to ATP provides an explanation for how these mutations lead to K_{ATP} channel overactivity in β-cells at high glucose, and thereby diabetes (20,21), some puzzles remain. For example, in some mutations, such as R201C and R201H, reduced ATP sensitivity was clearly observed in homomeric mutant channels; however, no significant difference in ATP sensitivity from wild-type channels could be detected under the condition that simulates heterozygous expression in patients (11,18,21). A potential explanation for the lack of detectable differ-

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CHOP, CIEBP homolog protein; DEND, developmental delay, epilepsy, and neonatal diabetes; K_{ATP} channel, ATP-sensitive K^+ channel; PNDM, permanent neonatal diabetes mellitus; SUR1, sulfonylurea receptor one; UPR, unfolded protein response.

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ence could be that the mutant is not expressed as efficiently as the wild-type Kir6.2, a hypothesis that has not been tested. Furthermore, the extent of ATP sensitivity reduction seen in the different mutant channels does not always match well with disease severity. For example, the mutation V59M results in channels much more sensitive to ATP than R201C or R201H, yet it is associated with more severe disease phenotype. These observations suggest additional factors might contribute to the pathogenic potency of a mutation.

Here, we examined the effects of several PNDM mutations, including Q52R, V59G, V59M, R201C, R201H, and I296L, on K_{ATP} channel expression. We found that all of them lead to reduced surface expression, to varying degrees, of K_{ATP} channels reconstituted in mammalian cells. By tagging the V59G or R201H mutant Kir6.2 with the N160D inward rectification mutation, which confers voltage-dependent spermine block of K_{ATP} channels (22), we demonstrate that in simulated heterozygous expression condition, nearly all channels present at the cell surface are either pure wild-type or heteromeric channels containing a mixture of wild-type and mutant Kir6.2. We present evidence that the expression level of a mutation plays a role in determining the extent of β-cell dysfunction. Moreover, we show that sulfonylureas significantly enhance surface expression of some PNDM mutant channels, suggesting the efficacy of sulfonylurea therapy may be compromised by its effect on channel expression.

RESEARCH DESIGN AND METHODS

Molecular biology. Rat Kir6.2 cDNA is in pCDNAI vector and SUR1 in pECE. Site-directed mutagenesis was carried out using the QuickChange site-directed mutagenesis kit (Strategene) and the mutation confirmed by sequencing. Construction of adenovirus carrying R201H Kir6.2 cDNA is as described previously (23).

Western blotting. Cells grown in 35-mm dishes were transfected using FuGene6 (Roche Applied Science, Indianapolis, IN) with 0.4 μg rat Kir6.2 and 0.6 μg of a SUR1 tagged with a FLAG-epitope (DYKDDDDK) at the NH_2-terminus (referred to as fSUR1) (24). Cells were lysed 48–72 h posttransfection in 20 mmol/l HEPES (pH 7.0, 5 mmol/l EDTA, 150 mmol/l NaCl, 1% Nonidet P-40 [Igapel]) with Complete protease inhibitors (Roche Applied Science) and lysate analyzed by standard Western blot procedures (24). Mouse monoclonal antibodies for FLAG (M2) and α-tubulin were from Sigma (St. Louis, MO), rabbit polyclonal antibodies for Kir6.2 and mouse monoclonal antibody for CIEBP homolog protein (CHOP) from Santa Cruz Biotechnology (Santa Cruz, CA), and mouse monoclonal antibody for BiP from BD Transduction Laboratories (San Diego, CA).

Chemiluminescence assay. COSm6 cells transiently expressing fSUR1 and Kir6.2 were fixed with 2% paraformaldehyde 48–72 h posttransfection, incubated with anti-FLAG antibody (10 μg/ml) followed by horseradish peroxidase–conjugated anti-mouse secondary antibodies (1:1,000 dilution; Amersham), and SuperSignal ELISA Femto luminol solution (24,25). Chemiluminescence signal was read in a TD-20/20 luminometer (Turner Designs). Results of each experiment are the average of 2–3 dishes, and each data point shown in figures is the average of 3–5 independent experiments.

Virus infection. INS-1 cells clone 832/13 (26) were plated in 24-well plates and cultured for 24 h in RPMI-1640 with 11.1 mmol/l D-glucose (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 mmol/l HEPES, 2 mmol/l glutamine, 1 mmol/l sodium pyruvate, and 50 μmol/l β-mercaptoethanol. Recombinant adenoviruses containing wild-type or R201H Kir6.2 with desired titers were then used to infect cells as described previously (23).

Insulin secretion assay. Insulin secretion assays in INS-1 cells were performed as described previously (23). Twenty-four hours post–virus infection, the culture medium was replaced by RPMI-1640 with 5 mmol/l glucose and cells incubated for 18 h. Insulin secretion was assayed by preincubating cells in Hanks' balanced salt solution (in mmol/l: 114 NaCl, 4.7 KCl, 1 $MgCl_2$, 1.2 KH_2PO_4, 1.16 $MgSO_4$, 20 HEPES, 2.5 $CaCl_2$, 25.5 $NaHCO_3$, and 0.2% BSA; pH ~7.2) (26) containing 3 mmol/l glucose for 2 h before stimulation with Hanks' balanced salt solution containing 3 or 12 mmol/l glucose for 2 h. Insulin content was determined using Immunochem coated-tube insulin radioimmunoassay from ICN Pharmaceuticals (Costa Mesa, CA). Insulin release at 12 mmol/l glucose was normalized to that at 3 mmol/l glucose and expressed as fold increase.

Electrophysiology. For experiments in Figs. 3 and 4 and the online appendix (availabe at http://diabetes.diabetesjournal.org), inside-out patch-clamp recording was performed in COSm6 cells expressing wild-type or mutant K_{ATP} channels. Recording pipettes had average resistance of ~1.0–1.5 MΩ. All recordings were made with the Axopatch 1D amplifier and Clampex 8.1 (Axon, Foster City, CA) at room temperature −50 mV with symmetrical K-INT solution containing (in mmol/l) 140KCl, 10 K-HEPES, and 1 KEGTA (pH 7.2 with KOH). For measuring ATP sensitivity, 1 mmol/l EDTA was included in K-INT to avoid channel rundown (27). For measuring MgATP sensitivity, $MgCl_2$ (at concentration equal to that of ATP) was added to K-INT as Mg^{2+} source. For spermine block experiments, 20 μmol/l spermine (Sigma) was added to K-INT. Excised patches were subjected to voltage ramps (200 ms) between +100 and −100 mV, with each ramp preceded by 20 ms holding potential at +140 mV to saturate channels with spermine (28). Currents obtained in the presence of 10 mmol/l ATP were taken as leakage and subtracted from the total currents for analysis. Only patches with currents >1 nA (at −50 mV) were included for analysis. Whole-cell patch-clamp recording was used to measure INS-1 cell resting membrane potential (23). Cells were preincubated in 12 mmol/l glucose for 3 h before recording. During recording, cells were bathed in Tyrode's solution consisting of (in mmol/l) 137 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 5 HEPES, 3 $NaHCO_3$, and 0.16 NaH_2PO_4, with 12 mmol/l glucose. Pipette solution contained (in mmol/l) 10 KCl, 130 Kgluconate, 10 HEPES, 1 EGTA, 3 $MgCl_2$, and 5 ATP.

Data analysis. Data fitting was performed with Origin 6.1. For estimating expression ratio of wild-type and N160D-tagged mutant subunit, the Grel/V curve obtained in the presence of spermine was fitted as described previously (28) with the sum of five individual Boltzmann equations:

$$G_{rel} = \Sigma_{i=1}^{5} A_i \cdot \{1 + \exp[(F/RT) \cdot z_i \cdot (V - V_i)]\}^{-1}$$

where A_i, V_i, and z_i are the amplitude, voltage of half-maximal inhibition, and effective valency, respectively, of the i^{th} component (with 0, 1, 2, 3, or 4 mutant subunits). We assumed that the fitted amiplitude (A_i) corresponds to the probability (p_x) of formation of each channel component, with wild-type and mutant Kir6.2 subunits having equal probability of being incorporated into the channel complex, following the binomial distribution:

$$p_x = \binom{n}{x} \cdot p^x \cdot (1 - P)^{n-x}$$

where n is the number of Kir6.2 subunits in a channel, x is the number, 0 to n, of wild-type subunits in a given channel component, P is the probability of inclusion of a wild-type subunit, and $(1 - P)$ is the probability of inclusion of a mutant subunit. The P value that gave the best fit was taken as the fraction of wild-type subunits present in the channel population in a given patch.

RESULTS

Effects of PNDM mutations on K_{ATP} channel expression. To study K_{ATP} channel expression, we chose the following Kir6.2 mutations: Q52R, V59G, V59M, R201C, R201H, and I296L to include both the mild PNDM disease phenotype and the more severe DEND phenotype. Although functional data reported to date has been largely obtained using the *Xenopus* oocyte expression system (11,18), this system is less suitable for studying channel maturation and trafficking since many misfolded proteins that fail to mature in mammalian cells are tolerated in *Xenopus* oocytes (29,30). We therefore examined channel expression in the mammalian cell line COSm6. Western blot analysis was used to assess steady-state channel protein expression level in cells cotransfected with Kir6.2 and fSUR1. Channel assembly occurs in the endoplasmic reticulum. As the correctly assembled channel complex exits the endoplasmic reticulum and travels through the Golgi, the two N-linked glycosylation sites in SUR1 are further modified, giving rise to the complex-glycosylated form that migrates slower on the SDS gel (the upper band) than the core-glycosylated form (the lower band). The intensity of the upper fSUR1 band is thus indicative of the level of assembled channel complexes that have trafficked beyond the medial Golgi. As shown in Fig. 1*A*, many of the mutants exhibited reduced upper fSUR1 band, indicating

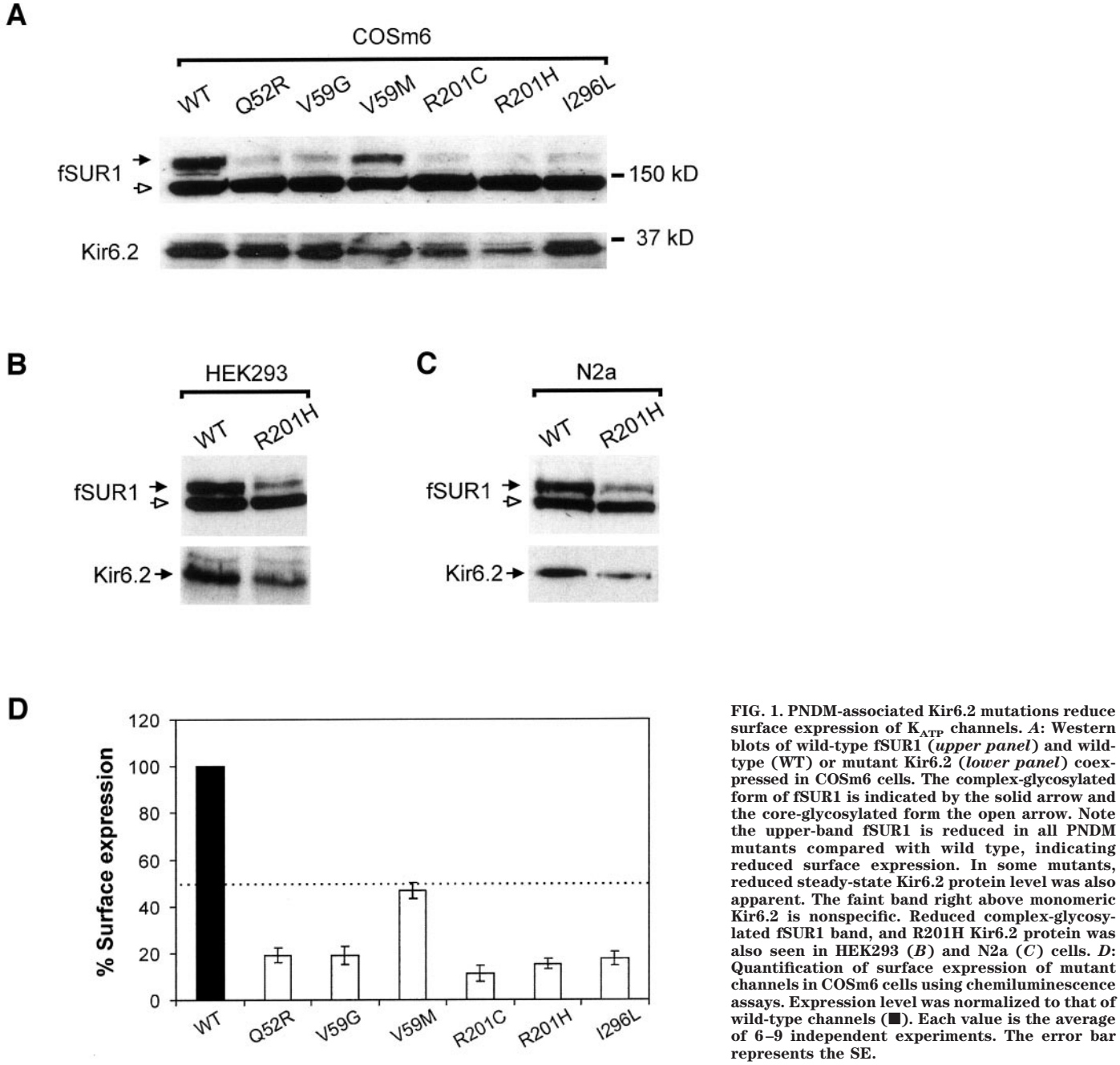

FIG. 1. PNDM-associated Kir6.2 mutations reduce surface expression of K_{ATP} channels. *A*: Western blots of wild-type fSUR1 (*upper panel*) and wild-type (WT) or mutant Kir6.2 (*lower panel*) coexpressed in COSm6 cells. The complex-glycosylated form of fSUR1 is indicated by the solid arrow and the core-glycosylated form the open arrow. Note the upper-band fSUR1 is reduced in all PNDM mutants compared with wild type, indicating reduced surface expression. In some mutants, reduced steady-state Kir6.2 protein level was also apparent. The faint band right above monomeric Kir6.2 is nonspecific. Reduced complex-glycosylated fSUR1 band, and R201H Kir6.2 protein was also seen in HEK293 (*B*) and N2a (*C*) cells. *D*: Quantification of surface expression of mutant channels in COSm6 cells using chemiluminescence assays. Expression level was normalized to that of wild-type channels (■). Each value is the average of 6–9 independent experiments. The error bar represents the SE.

reduced channel expression. In some mutants (for example, R201H) a reduced steady-state Kir6.2 protein level was also observed, presumably due to decreased synthesis or increased degradation; the reduced Kir6.2 protein level would lead to reduced channel expression. To ensure that the reduced expression is not unique to COSm6 cells, we next examined mutant channel expression in HEK293 cells and a neuroblastoma cell line N2a. Figure 1*B* and *C* show that the expression of the R201H mutant in these two cell lines was similarly reduced (other mutants also exhibited reduced expression as seen in COSm6 cells, not shown). Surface expression was further quantified using a chemiluminescence-based immunoassay (25,31). Consistent with Western blot results, mutant channels expressed in COS cells exhibited reduced surface expression to varying degrees, with Q52R, V59G, R201C, R201H, and I296L reduced by ~80% and V59M moderately reduced by ~50% (Fig. 1*D*).

One possible mechanism of reduced channel surface expression is that the mutant Kir6.2 proteins are unable to fold or assemble correctly. If such protein is not effectively degraded and accumulates in the endoplasmic reticulum, it may elicit unfolded protein response (UPR) and may even trigger apoptosis if the endoplasmic reticulum stress cannot be overcome (32). Although no apparent accumulation of Kir6.2 was seen for our mutants, we decided to test the UPR scenario directly since mutant protein-induced UPR and cell apoptosis could be a contributing mechanism to neonatal diabetes. We examined two marker proteins, BiP (GRP78), an endoplasmic reticulum chaperone protein, and CHOP (GADD153), an endoplasmic reticulum stress-associated apoptosis transcription factor, whose levels are known to be upregulated during UPR and UPR-induced apoptosis (33). No difference in either BiP or CHOP protein level was detected between

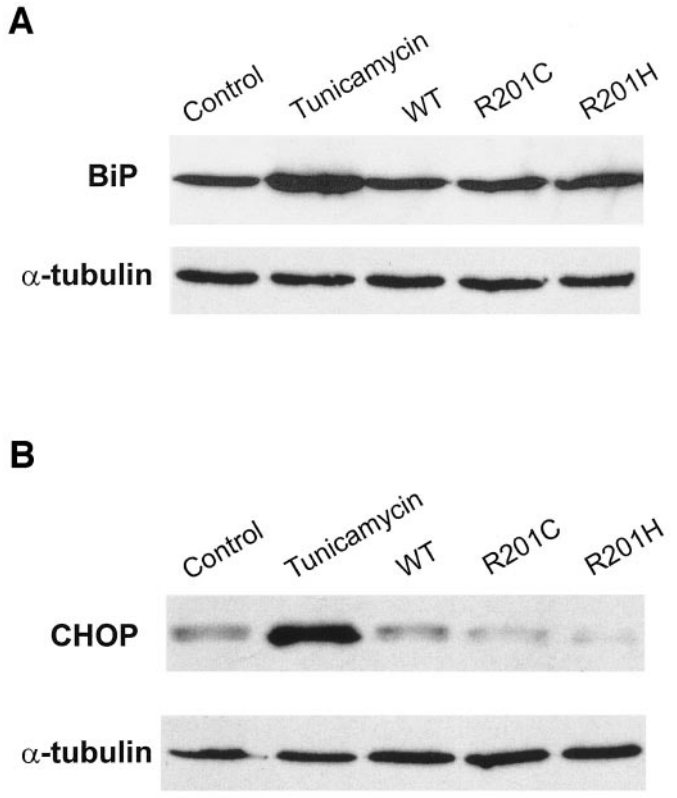

FIG. 2. Expression of mutant Kir6.2 in COSm6 cells does not induce unfolded protein response or apoptosis. Western blots of BiP (*A*) or CHOP (*B*) from COSm6 cells expressing wild-type (WT) or mutant K_{ATP} channel proteins (wild-type SUR1 plus wild-type or mutant Kir6.2). Cells treated with tunicamycin (1 μg/ml for 8 h), a drug known to induce the expression of BiP and CHOP, were included as positive control. The same blots were probed for α-tubulin to show that the amount of protein loaded in each lane was similar.

control COSm6 cells, cells expressing wild-type K_{ATP} channels and cells expressing PNDM mutant channels (Fig. 2). In contrast, cells treated with tunicamycin, which blocks N-linked protein glycosylation and induces UPR, exhibited increased level of both BiP and CHOP (34). These results indicate that the mutant Kir6.2 proteins do not cause significant endoplasmic reticulum stress and apoptosis, at least in COSm6 cells, within the time course of our experiments.

Assessing the relative abundance of wild-type and mutant Kir6.2 in surface channels in simulated heterozygous expression by N160D mutation-dependent and voltage-dependent spermine block. The reduced surface expression of the mutant channels observed above suggests that under heterozygous expression condition as seen in patients few surface channels would be pure (homomeric) mutant channels. To test this, we monitored the relative number of surface channels containing 0, 1, 2, 3, or all 4 mutant subunits in heterozygous expression by tagging the mutant Kir6.2 subunits with the N160D mutation, which confers strong inward rectification in the presence of spermine (22,28). Previous studies have shown that the extent of rectification depends on the number of Kir6.2 subunits carrying the N160D mutation and that the relative conductance-voltage (Grel-V) curve of each channel population containing 0, 1, 2, 3, or 4 N160D subunits can be fitted by a single Boltzmann function (22). In simulated heterozygous expression, the overall Grel-V curve will therefore be the sum of the five individual Boltzmann functions with the relative conductance amplitude of each Boltzmann function corresponding to the relative abundance of each channel population (28). Assuming wild-type and mutant Kir6.2 protein have equal probability of being incorporated into the channel complex, the distribution of the five-channel populations should be binomial with the relative abundance of each channel population dictated by the relative amount of the wild-type versus mutant Kir6.2 proteins. This approach can thus be used to estimate the ratio of wild-type to mutant Kir6.2 in a single membrane patch. In cells expressing wild-type and R201H/N160D Kir6.2 at 1:1 cDNA ratio, the patches consistently contained ~83% of wild-type protein and ~17% of R201H/N160D protein ($n = 7$), in good agreement with biochemical data that R201H Kir6.2 is not equally expressed as wild-type Kir6.2 (see Fig. 1). Control experiments confirmed that the N160D mutation does not affect expression of the R201H channels (not shown). Decreasing cDNA ratio of wild-type to mutant Kir6.2 to 1:2 or 1:4 resulted in concordant change in the estimated protein ratio (wild-type–to–mutant ratio of 0.72:0.28 for cDNA ratio of 1:2, $n = 6$, and 0.60:0.40 for cDNA ratio of 1:4, $n = 7$), as evidenced by the more severe inward-rectification in intracellular 20 μmol/l spermine (an example of 1:4 cDNA ratio expression experiment is shown in Fig. 3). Thus, in simulated heterozygous state, although the cDNA ratio of wild type to mutant is 1:1, the protein ratio is ~1:0.2. These results led us to conclude that in simulated heterozygous expression, most surface channels are either pure wild type or contain a mixture of wild-type and R201H mutant Kir6.2 subunits, with the population of homR201H mutant channels being <0.1% [$(0.17)^4$]. Examination of cells coexpressing wild-type and V59G/N160D Kir6.2 at 1:1 cDNA ratio in the spermine block experiments similar to those described for R201H also confirmed reduced expression of the V59G mutant in the surface membrane (wild-type–to–V59G/N160D protein ratio of 0.83:0.17, $n = 7$). The above functional data further corroborate the results from Western blots and chemiluminescence assays that many PNDM mutations render reduced surface expression of K_{ATP} channels.

Heterozygous expression of R201H results in channels that are overactive in physiological concentrations of MgATP. If only <0.1% of homR201H mutant channels are expressed at the cell surface, how could heterozygous R201H mutation increase the physiological activity of β-cell K_{ATP} channels? We reasoned that in cells, most ATP is complexed with Mg^{2+} (35). In the presence of Mg^{2+}, ATP is hydrolyzed by SUR1; this leads to channel activation and effectively reduces the sensitivity of Kir6.2 to ATP inhibition (1,4,8). We therefore tested whether heterozygous R201H mutation might significantly affect channel sensitivity to MgATP. Indeed, we found that in simulated heterozygous expression (cDNA ratio of wild type to mutant = 1:1, i.e., protein ratio of ~0.83:0.17 as shown in Fig. 3*B*), the resulting channel population exhibits significantly increased channel activity in mmol/l range of MgATP compared with pure wild-type channels (Fig. 4*A*). These observations are consistent with those recently reported by Gloyn et al. (36) and Proks et al. (37). Since the number of pure R201H mutant channels is close to 0 in heterozygous expression, based on the conservative estimation that the number of surface K_{ATP} channels in a single human β-cell is <800 (38,39), we conclude that the active channels in mmol/l MgATP concentration range are

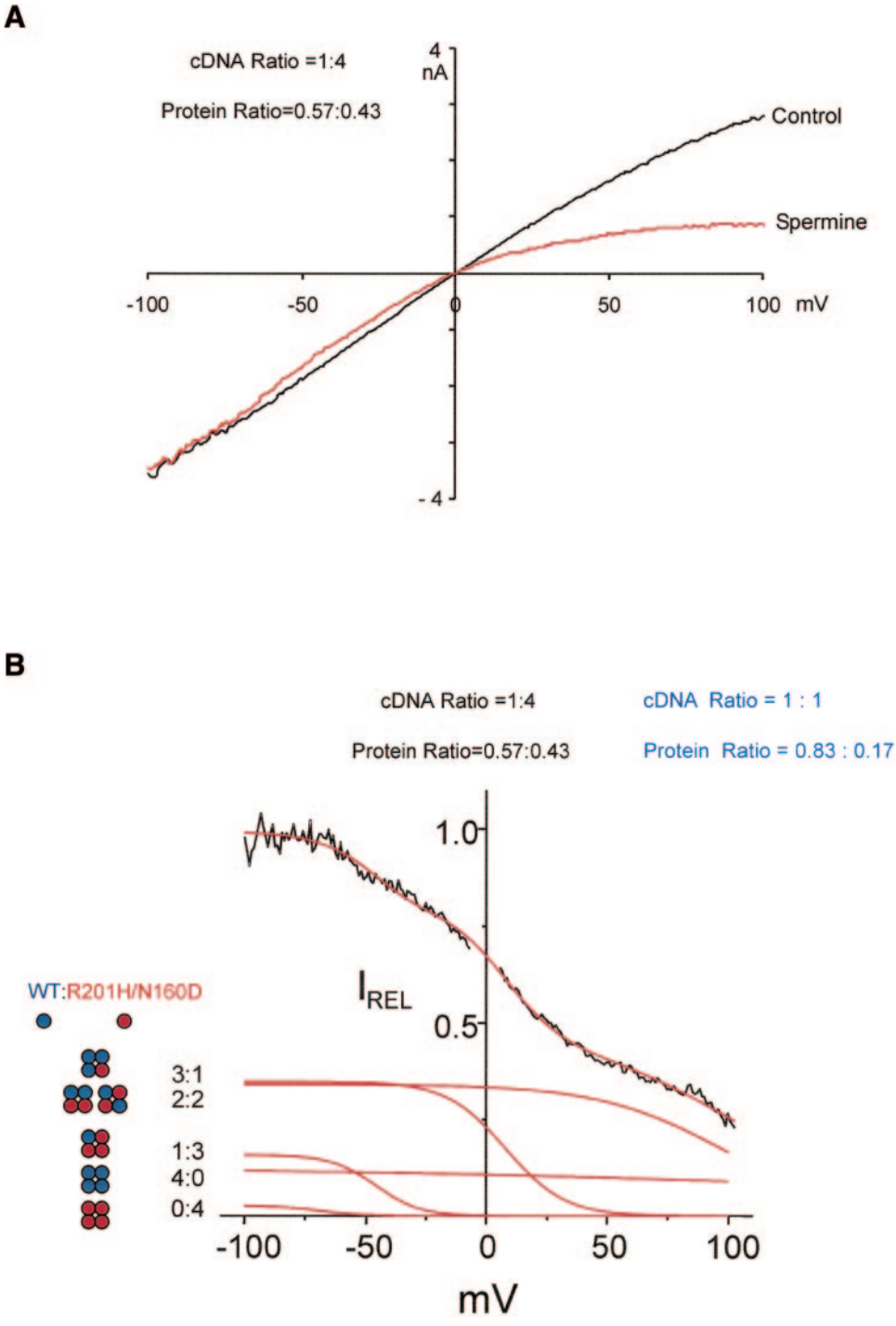

FIG. 3. Monitoring the relative abundance of wild-type and R201H mutant Kir6.2 subunits in surface K_{ATP} channels in simulated heterozygous expression using voltage-dependent spermine block. R201H mutant Kir6.2 was tagged with the N160D mutation, which confers channel sensitivity to voltage-dependent spermine block. *A*: Current-voltage relationships of K_{ATP} channels in the absence (control) or presence (20 μmol/l) of spermine. *B*: Relative conductance (Grel)-voltage relationships from the data shown in *A*. Note for illustration purposes, the curves presented in this figure (in both *A* and *B*) are from an experiment where wild-type and R201H/N160D Kir6.2 were coexpressed at a cDNA ratio of 1:4. At this cDNA ratio, the protein ratio is close to 1:1 (0.57:0.43 for the patch shown) such that the five different channel populations (shown as the five individual Boltzmann function plots with the contributing number of wild-type and mutant subunits indicated next to each curve) can be clearly seen. In *B*, the smooth red line superimposed on the data (shown in black) is the sum of five Boltzmann functions that correspond to different combinations of wild-type and mutant Kir6.2 in a tetramer. The value of protein ratio calculated by fitting the overall Grel-V curve is shown on the top. At 1:4 cDNA ratio, a protein ratio of 0.57:0.43 gives the best fit. At 1:1 cDNA ratio, a protein ratio of 0.83:0.17 gives the best fits (in blue), consistent with estimation from the Western blots and chemiluminescence surface expression assay shown in Fig. 1.

those containing 1, 2, or 3 R201H mutant Kir6.2 subunits (Fig. 4*B*).

Effect of R201H-Kir6.2 expression level on INS-1 cell membrane potential and insulin secretion response. A question that arises from our observation of the varied expression efficiency of PNDM mutants (Fig. 1*D*) is whether the expression level plays a role in determining the extent of β-cell dysfunction. To address this issue, we expressed the R201H-Kir6.2 mutant in the insulin-secreting cell line INS-1 by infection with a recombinant adenovirus containing R201H-Kir6.2 cDNA. We chose two virus titers that gave one- or threefold excess of mutant protein expression compared with endogenous wild-type Kir6.2 protein (Fig. 5*A*). To ensure that cells expressing the mutant protein were not under severe endoplasmic reticulum stress or undergoing apoptosis, which could reduce insulin secretion, we again checked the level of BiP and CHOP. Similar to what we found in COS cells (Fig. 2), no change in either BiP or CHOP was detected during the time course of our experiments (Fig. 5*B*). Next, membrane potential and insulin secretion response to glucose stimulation (12 mmol/l) were measured by whole-cell patch-clamp recording and insulin radioimmunoassay. Figure 5*C* shows that the membrane potential of cells infected with R201H-Kir6.2 at 12 mmol/l glucose was more hyperpolarized, in an expression level–dependent manner, than uninfected controls cells or cells expressing similar levels of exogenous wild-type Kir6.2. Moreover, cells infected with

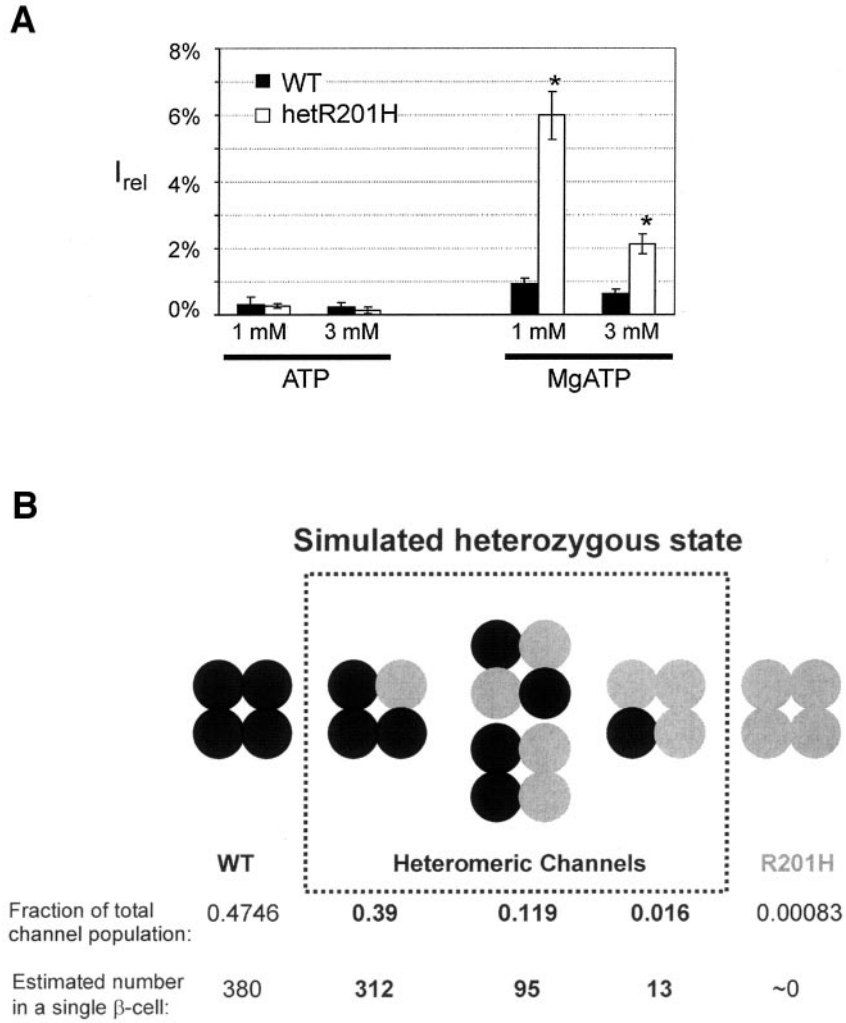

FIG. 4. Reduced sensitivity of hetR201H channels to physiological concentrations of MgATP. *A*: Comparison of channel activity from wild-type (WT) and hetR201H channels in 1 or 3 mmol/l ATP in the absence or presence of Mg^{2+} (MgATP). Currents were normalized to that observed in the absence of ATP (I_{rel}). *B*: Schematic of the fraction of the different channel types expected when wild-type and R201H Kir6.2 are expressed at equal cDNA ratio (simulated heterozygous state) and the corresponding number of each type of channel expected in the plasma membrane of a single β-cell. Although homR201H channels are very active in physiological concentrations of MgATP (see online appendix Fig. 2), they are unlikely to contribute to K_{ATP} channel conductance in β-cells since they are practically nonexistent in the heterozygous state. Rather, the overactivity of K_{ATP} channels seen in patients is likely due to heteromeric channels containing 1, 2, or 3 mutant subunits. Estimated fraction of individual channel populations (0, 1, 2, 3, or 4 mutants) in the total channel population is based on the wild-type–to–R201H protein ratio of 0.83:0.17 as shown in Fig. 3*B*. The number of each type of channels in a single β-cell is calculated assuming the total number of K_{ATP} channels is 800 (a conservative overestimation based on the literature) (39,49).

the R201H-Kir6.2 virus had reduced insulin secretion response to 12 mmol/l glucose stimulation, again in an expression level–dependent manner (Fig. 5*D*). These results demonstrate directly the causal role of the R201H mutation in impairing glucose-stimulated insulin secretion. In addition, they provide evidence that the expression level of a PNDM mutant Kir6.2 indeed affects the extent of β-cell dysfunction.

Effects of sulfonylureas on surface expression of PNDM mutant channels. Sulfonylurea therapy has been proposed as an alternative to insulin injection in PNDM patients with Kir6.2 mutations. We have previously shown that sulfonylureas increase surface expression of mutant channels with reduced surface expression due to SUR1 mutations associated with congenital hyperinsulinism (24,25). We asked whether sulfonylureas might also affect the expression efficiency of PNDM mutant channels. Interestingly, surface expression of several mutants in COSm6 cells was markedly increased when cells were treated with glibenclamide (1 μmol/l for 24 h; Fig. 6). The degree of increase appeared more pronounced in mutants with more severe expression defect. These results reveal the complex effects of sulfonylureas on not only gating but also expression of PNDM mutants.

DISCUSSION

In this study, we used both biochemical and electrophysiological approaches to demonstrate that many PNDM-associated Kir6.2 mutations reduce K_{ATP} channel surface expression, in addition to their well-documented effects on reducing channel ATP sensitivity (11–13,18,36,40). Detailed analyses of the R201H mutation show that in simulated heterozygous state the fraction of mutant Kir6.2 present in the plasma membrane is only ~17% of the total surface Kir6.2 protein pool. The findings suggest that in patients, the number of homomeric mutant channels in a β-cell is almost zero and overactivity of K_{ATP} channels is therefore likely attributed to heteromeric channels containing both wild-type and mutant Kir6.2 subunits (Fig. 4). Interestingly, earlier studies showed that while reduced ATP sensitivity was obvious in homomeric R201H mutant channels, no significant change in ATP sensitivity was detectable in channels from simulated heterozygous expression condition. Our observation that R201H homomeric channels are poorly expressed (or nonexistent) at the cell surface may help explain why little change in channel ATP sensitivity was observed under heterozygous expression (11,18,21). The R201 residue is thought to be involved in ATP binding (40,41). Markworth et al. (42) have reported that only one of the four ATP binding sites in a Kir6.2 tetramer is necessary for ATP-induced channel closure; the wild-type subunit in heteromeric channels may thus be sufficient to confer wild-type–like ATP sensitivity. This interpretation, however, may not apply to other mutations, such as Q52R and V59G, that render the channel insensitive to ATP by increasing the intrinsic channel

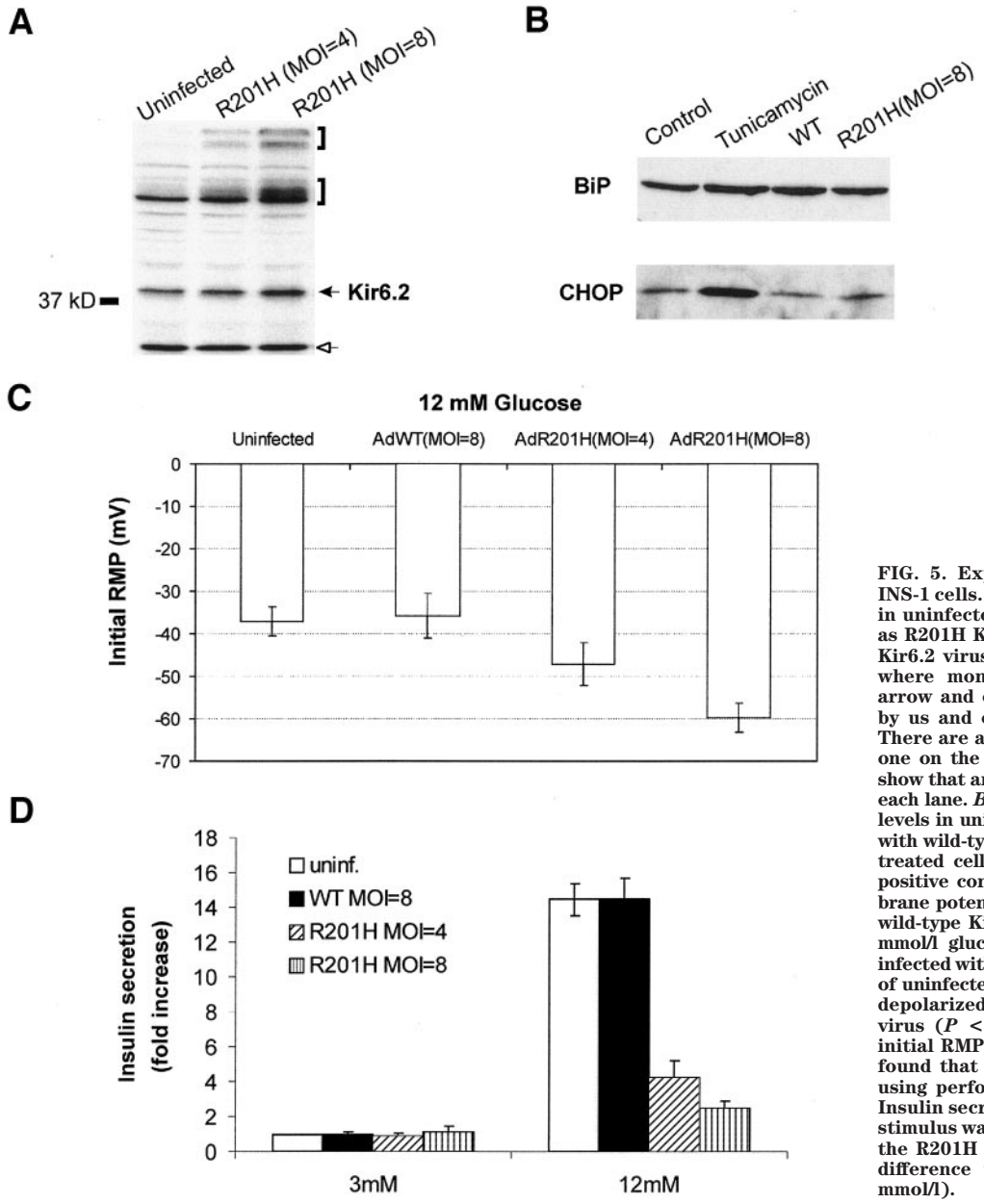

FIG. 5. Expression of R201H mutant Kir6.2 in INS-1 cells. *A*: Western blots of endogenous Kir6.2 in uninfected INS-1 cells and endogenous as well as R201H Kir6.2 in cells infected with the R201H Kir6.2 virus. Shown is the full length of the blot where monomeric Kir6.2 is indicated by solid arrow and oligomeric Kir6.2 previously reported by us and others indicated by brackets (24,50). There are a number of nonspecific bands; a major one on the bottom is indicated by open arrow to show that an equal amount of protein was loaded in each lane. *B*: Comparison of BiP and CHOP protein levels in uninfected control cells and cells infected with wild-type or R201H Kir6.2 virus. Tunicamycin-treated cells (1 μg/ml for 4 h) were served as a positive control (see Fig. 2). *C*: The resting membrane potential (RMP) of INS-1 cells infected with wild-type Kir6.2 virus or R201H Kir6.2 virus at 12 mmol/l glucose. (n = 12–21). The RMP of cells infected with wild-type Kir6.2 virus is similar to that of uninfected cells (23) and is significantly more depolarized than cells infected with the R201H virus (P < 0.01). Note the value shown is the initial RMP upon break in. Previous studies have found that this value is similar to that obtained using perforated patch-clamp recording (23). *D*: Insulin secretion in response to 12 mmol/l glucose stimulus was greatly reduced in cells infected with the R201H Kir6.2 virus (P < 0.001), whereas no difference was seen at basal level glucose (3 mmol/l).

open probability rather than affecting ATP binding (18). In these mutants, it is possible that even one mutant subunit in the tetramer can increase channel open probability and indirectly reduce ATP sensitivity. Although heterozygous expression of R201H does not lead to significant reduction in ATP sensitivity, it does lead to significantly higher channel activity in physiological concentration of MgATP (Fig. 4*A*), consistent with that reported recently by others (36,37). Our analyses of other mutants also showed more pronounced channel overactivity in MgATP than in ATP (see online appendix). Collectively, these results lead us to conclude that for mutations presented in this study, the heteromeric channel population containing both wild-type and mutant Kir6.2 subunits plays a dominant role (as opposed to homomeric mutant channels) in causing disease by being more active in physiological concentration of MgATP.

Channel expression and disease. Reduced surface expression of K_{ATP} channels due to mutations in the channel genes has been recognized as a significant mechanism contributing to loss of channel function in congenital hyperinsulinism (3,43–45). Somewhat unexpectedly, all of the PNDM mutations examined here also reduce channel surface expression to varying degrees. While intuitively one might predict that reduced surface expression should lead to loss of channel function, in the case of PNDM mutations the effect on expression is overruled by the effect of the mutations on gating (increased channel open probability in MgATP), leading to an overall "gain-of-channel-function" phenotype. This is not surprising considering that the activity of K_{ATP} channels in cells depends on both the number of channels in the plasma membrane and the open probability of channels under the physiological environment. Our finding also raises the question of whether the expression level of a PNDM mutation plays a role in its clinical manifestation. In this regard, our study examining the effect of R201H-Kir6.2 expression in INS-1 cells demonstrates that indeed the extent of β-cell dysfunction, as assessed by the ability of the cell to depolarize and secrete insulin upon glucose stimulation, is correlated

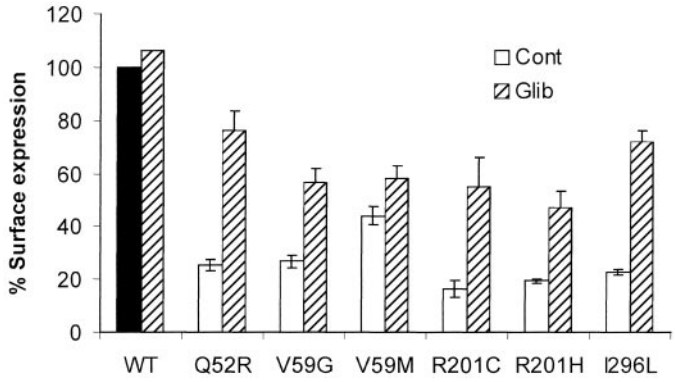

FIG. 6. Effects of sulfonylureas on expression of PNDM-causing mutant K_{ATP} channels. Surface expression of various PNDM mutant channels in cells treated with (Glib) or without (Cont) 1 μmol/l glibenclamide for 24 h was quantified by chemiluminescence assays and normalized to that of wild-type (WT) channels without glibenclamide treatment (black bar). Glibenclamide treatment increased surface expression of all mutants to varying degrees. Each bar represents means ± SE of 4–5 experiments.

with the expression level of the mutant. Among the PNDM mutations we examined, V59M stands out as having the highest expression level (Fig. 1). It is interesting to note that V59M channels exhibit the least change in ATP and MgATP sensitivities (Figs. 1 and 2; Tables 1 and 2, online appendix), yet it is associated with intermediate DEND that is more severe than the PNDM phenotype seen in another mutation, R201H, which causes much more reduced ATP sensitivity. We recognize that many factors are likely involved in determining the clinical phenotypes in patients; for instance, the R201C mutation has been reported to cause PNDM in some individuals but DEND in others (11,12,14). Nevertheless, it is tempting to speculate that the more severe phenotype seen in the V59M patients might be due in part to the mutant's higher expression level (Table 2, online appendix).

Implications for sulfonylurea therapy. The discovery that PNDM could be caused by overactive K_{ATP} channels has led to the proposal that sulfonylureas might be used as an alternative therapy to insulin injection in patients carrying Kir6.2 mutations (11). Indeed, several studies have reported successful treatment of patients with mutations that cause PNDM alone using sulfonylureas (11,13,46,47), although the long-term effectiveness of such treatment remains to be determined. We have previously shown that sulfonylureas markedly increase surface channel expression in two congenital hyperinsulinism-causing SUR1 mutations (25). In this study, we found that sulfonylureas also markedly increase surface expression level of several PNDM-associated mutant channels. Thus, on the one hand, sulfonylureas might reduce channel activity via their effects on channel gating; on the other, they might have the adverse effect of increasing channel activity by increasing mutant channel expression. Our finding, together with previous studies showing that some PNDM Kir6.2 mutations render the channel less sensitive to sulfonylurea inhibition (18,48), point to the potential complexity of sulfonylurea therapy. Future studies examining the effect of sulfonylureas on INS-1 cells expressing mutant channels may help evaluate the effectiveness of sulfonylurea therapy in specific mutations.

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