Sulfonylurea Receptor 1 Mutations That Cause Opposite Insulin Secretion Defects with Chemical Chaperone Exposure

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The β-cell ATP-sensitive potassium (K\textsubscript{ATP}) channel composed of sulfonylurea receptor SUR1 and potassium channel Kir6.2 serves a key role in insulin secretion regulation by linking glucose metabolism to cell excitability. Mutations in SUR1 or Kir6.2 that decrease channel function are typically associated with congenital hyperinsulinism, whereas those that increase channel function are associated with neonatal diabetes. Here we report that two hyperinsulinism-associated SUR1 missense mutations, R74W and E128K, surprisingly reduce channel inhibition by intracellular ATP, a gating defect expected to yield the opposite disease phenotype neonatal diabetes. Under normal conditions, both mutant channels showed poor surface expression due to retention in the endoplasmic reticulum, accounting for the loss of channel function phenotype in the congenital hyperinsulinism patients. This trafficking defect, however, could be corrected by treating cells with the oral hypoglycemic drugs sulfonylureas, which we have shown previously to act as small molecule chemical chaperones for K\textsubscript{ATP} channels. The R74W and E128K mutants thus rescued to the cell surface paradoxically exhibited ATP sensitivity 6- and 12-fold lower than wild-type channels, respectively. Further analyses revealed a nucleotide-independent decrease in mutant channel intrinsic open probability, suggesting the mutations may reduce ATP sensitivity by causing functional uncoupling between SUR1 and Kir6.2. In insulin-secreting cells, rescue of both mutant channels to the cell surface led to hyperpolarized membrane potentials and reduced insulin secretion upon glucose stimulation. Our results show that sulfonylureas, as chemical chaperones, can dictate manifestation of the two opposite insulin secretion defects by altering the expression levels of the disease mutants.

The β-cell ATP-sensitive potassium (K\textsubscript{ATP})\textsuperscript{3} channels are essential for triggering glucose-stimulated insulin secretion as they couple metabolic signals to electrical signals (1). Each channel is a complex of four regulatory sulfonylurea receptor 1 (SUR1) subunits, encoded by ABCG8, and four pore-forming Kir6.2 inwardly rectifying potassium channel subunits, encoded by KCNJ11 (1). Mutations in ABCG8 or KCNJ11 that perturb the expression and/or gating of the channel lead to channel dysfunction and insulin secretion disorders. Whereas a net loss in channel activity results in congenital hyperinsulinism (CHI), a net gain of channel function causes permanent neonatal diabetes mellitus (PNDM) (2).

SUR1 and Kir6.2 co-assemble in the endoplasmic reticulum (ER) to form channel complexes; successful assembly overcomes the arginine-lysine-arginine (RKR) ER retention motif in SUR1 and Kir6.2 to permit channel trafficking to the plasma membrane (3, 4). Quality surveillance mechanisms are in place to prevent misfolded or unassembled channel subunits from exiting the ER where the retained proteins are eventually degraded by the ubiquitin-proteasome pathway (5). In the plasma membrane, channel activities are regulated by intracellular ATP and MgADP (1). ATP inhibits channel activity by binding to Kir6.2, whereas MgADP stimulates channel activity by interacting with the nucleotide binding domains (NBDs) of SUR1 (6). A Kir6.2 C-terminal truncation mutant lacking the last 36 amino acids and the -RKR- ER retention signal (Kir6.2ΔC) can form channels that are still sensitive to ATP inhibition even in the absence of SUR1 (7). However, the ATP sensitivity of Kir6.2ΔC channels is substantially lower than wild-type (WT) channels, indicating SUR1 hypersensitizes Kir6.2 to ATP inhibition (7, 8). Furthermore, SUR1 markedly increases the intrinsic open probability (P\textsubscript{o}) of Kir6.2 channels in the absence of intracellular nucleotides (6). Recent studies have shown that the first transmembrane domain of SUR1, known as TMD0, is sufficient to increase the P\textsubscript{o} of Kir6.2ΔC channels (9, 10). Interestingly, TMD0 further reduces the ATP sensitivity of the Kir6.2ΔC channel, suggesting other SUR1

\textsuperscript{3}The abbreviations used are: K\textsubscript{ATP}, ATP-sensitive potassium channel; SUR1, sulfonylurea receptor 1; CHI, congenital hyperinsulinism; PNDM, permanent neonatal diabetes mellitus; ER, endoplasmic reticulum; P\textsubscript{o}, open probability; TMD, transmembrane domain; WT, wild type; HBSS, Hanks’ balanced salt solution; NBD, nucleotide binding domain; PBS, phosphate-buffered saline; BSA, bovine serum albumin; RMP, resting membrane potential.

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\textsuperscript{6}The on-line version of this article contains supplemental Figs. S1–S3.
domains are necessary to hypersensitize Kir6.2 channels to ATP inhibition (9, 11). Precisely how TMD0 couples to Kir6.2 to modulate channel gating remains to be elucidated.

Characterized by persistent insulin secretion despite severe hypoglycemia in neonates and infants, CHI frequently results from loss of function K\textsubscript{ATP} channel mutations (12). These mutations typically reduce channel activity by preventing channel expression at the cell surface, diminishing channel response to MgADP stimulation and/or reducing channel sensitivity to ATP inhibition or enhanced channel response to MgADP stimulation (2). We have previously shown that channel trafficking defects caused by CHI mutations in the TMD0 of SUR1 can be overcome by the oral hypoglycemic drugs sulfonylureas, which act as chemical chaperones to increase the biogenesis efficiency of these mutant channels (13–15). Here, we report two mutants, R74W and E128K, which, upon rescue to the cell surface, surprisingly revealed reduced ATP sensitivity-gating defects. Such defects are typically associated with PNDM mutations. Indeed, sulfonylurea-rescued surface expression of these two channels in insulin-secreting cells caused hyperpolarized membrane potentials and blunted insulin secretion in high glucose. Interestingly, unlike previously reported ATP-insensitive mutants, which tend to have increased intrinsic $P_v$, the R74W and E128K mutants showed reduced intrinsic $P_v$. The finding suggests R74W and E128K diminish channel ATP sensitivity by a distinct mechanism that likely involves functional uncoupling between SUR1 and Kir6.2.

**MATERIALS AND METHODS**

**Molecular Biology**—Rat Kir6.2 cDNA is in pCDNAI/Amp vector and SUR1 or N terminus FLAG-epitope (DYKDDDDK)-tagged SUR1 (fSUR1) in pECE (16). Site-directed mutagenesis was performed using the QuikChange mutagenesis kit (Stratagene), and mutations were confirmed by sequencing. SUR1 and Kir6.2 recombinant adenoviruses were constructed using the AdEasy kit (Stratagene) as described previously (15, 17).

**Virus Infection and Insulin Secretion Assay**—INS-1 cells (clone 832/13) were cultured in RPMI 1640 with 11.1 mM D-glucose plus 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol (18). For iSUR1 and Kir6.2 expression, recombinant viruses containing WT or mutant iSUR1 and WT Kir6.2 with desired titers were used to infect cells as described previously (19). Cells at ~50% confluency were washed with PBS and incubated for 90 min at 37 °C in Opti-MEM (Invitrogen) containing a mixture of viruses each with a multiplicity of infection that yielded desired protein expression levels. At the end of the incubation, 2× growth medium was added, and the cells were cultured at 37 °C until experiments.

Insulin secretion assays were performed as described previously (17). INS-1 cells seeded in 24-well tissue culture plates at ~5 × 10\textsuperscript{5}/well were infected with viruses as described above. Twenty-four hours post-infection, the culture medium was replaced by RPMI 1640 with 5 mM glucose, and cells were incubated for at least 18 h. Cells were treated with 300 μM tolbutamide during the last 4 h of incubation to rescue mutant channel surface expression. Insulin secretion was assayed in HEPES balanced salt solution buffer consisting of (in mM) 114 NaCl, 4.7 KCl, 1 MgCl\textsubscript{2}, 1.2 KH\textsubscript{2}PO\textsubscript{4}, 1.16 MgSO\textsubscript{4}, 20 HEPES, 2.5 CaCl\textsubscript{2}, 25.5 NaHCO\textsubscript{3}, and 0.2% bovine serum albumin (pH ~ 7.2). Cells were washed twice with pre-warmed (37 °C) HEPES buffer with 3 mM glucose followed by 2-h incubation in the same buffer prior to stimulation with 0.8 ml/well pre-warmed HEPES buffer containing 3 or 12 mM glucose for 2 h. The medium was harvested, and the amount of insulin was determined using Immunochem-coated tube insulin radioimmunoassay from ICN Pharmaceuticals (Costa Mesa, CA). Insulin content in the medium was divided by the total cellular insulin content to correct for the number of cells. The resulting value was then normalized to that observed in uninfected cells at 3 mM glucose and expressed as -fold increase in insulin secretion.

**Rat Pancreatic Islet Preparation**—Animals were treated in accordance with institutional regulations. Rats aged 6–8 weeks were injected with 5 ml of cold Hanks’ balanced salt solution (HBSS, Invitrogen) containing 0.3 mg/ml Liberace R1 (Roche Applied Science) from the common bile duct, the distant junction to intestine was completely blocked by clamping. Pancreas were then dissected and minced before incubating in a total volume of 10 ml of HBSS at 37 °C for 30 min to allow digestion to occur. Digestions were stopped with the addition of 45 ml of HBSS containing 10% fetal bovine serum. Tissue suspensions were centrifuged at 1000 rpm for 1 min, supernatants were removed, and the pellets were resuspended in 50 ml of HBSS plus 10% fetal bovine serum. Pancreatic islets were visualized by adding 0.5 mg/ml diphenylthiocarbazone (Sigma) and were placed individually into fresh HBSS and then into RPMI media containing 5 mM glucose, 10% fetal bovine serum, and penicillin/streptomycin supplements. Approximately 400 islets were maintained for 48 h at 37 °C in a humidified chamber with 5% CO\textsubscript{2} before infection with viruses. Virus infection was carried out as in INS-1 cells described above. About 100 islets matched in size were used for each infection. The multiplicity of infection of each adenovirus was 10-fold higher than that used for INS-1 cells to achieve the desired protein expression level. Infected islets were treated with sulfonylureas the following day for 12 h and then harvested with lysis buffer for Western blot as described below.

**Immunoblotting, Immunostaining, and Chemiluminescence Assays**—INS-1 cells or rat islets infected with iSUR1 and Kir6.2 viruses were treated with or without glibenclamide (5 μM) or tolbutamide (300 μM) for 12–24 h before being processed for immunoblotting or immunofluorescent staining as described previously (13, 15). For immunoblotting, cells were lysed in 20 mM HEPES, pH 7.0/5 mM EDTA/150 mM NaCl/1% Nonidet P-40 with Complete\textsuperscript{TM} protease inhibitors (Roche Applied Science) 48–72 h post-transfection or infection. Proteins were separated by SDS-PAGE (8%), transferred to nitrocellulose, analyzed by M2 anti-FLAG antibody (Sigma) and then washed with antibodies conjugated to mouse secondary antibodies (Amersham Biosciences), and visualized by chemiluminescence (Super Signal West Femto, Pierce). For surface staining, living cells were incubated with anti-FLAG M2 mouse monoclonal antibody (10 μg/ml in Opti-MEM containing 0.1% bovine serum albumin.
(BSA)) for 1 h at 4°C, washed with ice-cold PBS, then fixed with cold (−20°C) methanol for 10 min and incubated with Cy-3 conjugated donkey anti-mouse secondary antibodies (Jackson) for 30 min at room temperature. After 3 × 10 min washes in PBS/0.1% BSA and 1 × 10 min wash in PBS, cells were viewed with an Olympus Fluoview confocal microscope. For chemiluminescence assays, cells were fixed with 2% paraformaldehyde for 30 min at 4°C, preblocked in PBS/0.5% BSA for 30 min, incubated in M2 anti-FLAG antibody (10 µg/ml) for an hour, washed 4 × 30 min in PBS/0.5% BSA, incubated in HRP-conjugated anti-mouse antibodies for 20 min, and washed again 4 × 30 min in PBS/0.5% BSA. Chemiluminescence was quantified using a TD-20/20 luminometer (Turner Designs) following 5-s incubation in Power Signal ELISA Femto luminol solution (Pierce). All steps after fixation were performed at room temperature. Results of each experiment are the average of two dishes, and each data point shown in the figure is the average of three experiments.

**Patch Clamp Recordings**—Inside-out patch clamp recordings in COSm6 and INS-1 cells were performed using an Axopatch 1D amplifier and pClamp9 acquisition software (Axon Inc.) (13). Micropipettes were pulled from non-heparinized Kimble glass on a horizontal puller (Sutter Instrument). The bath and pipette solution (K-INT) was: 140 mM KCl, 10 mM K-HEPES, 1 mM K-EGTA, pH 7.4, and pipette resistance was ~1.5 MΩ. For ATP dose-response experiments, 1 mM EDTA was added to K-INT to prevent channel rundown (20). For MgADP stimulation, free Mg²⁺ concentration was 1 mM. All currents were measured at −50 mV membrane potential at room temperature. Whole cell patch clamp recording was used to measure INS-1 cell membrane potential (19). One day after virus infection, INS-1 cells were preincubated in 5 mM glucose RPMI medium for 18 h followed by incubation in 12 mM glucose for 3 h before recording (17, 19). During recording, cells were bathed in Tyrode’s solution consisting of (in mM): 137 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.5 MgCl₂, 5 HEPES, 3 NaHCO₃, 0.16 NaH₂PO₄, with 12 mM glucose. Pipette solution contained (in mM): KCl, 130 K⁺-gluconate, 10 HEPES, 1 EGTA, 3 MgCl₂, and 5 ATP.

**Data Analysis**—ATP dose-response curve fitting was performed with Origin6.1. Single channel open probability (Pₒ) analysis was carried out using the pCLAMP9 automated channel-event detector and via amplitude histogram examination. Briefly, data collected within the first 90 s of inside-out patch excision were binned by amplitude using 0.05-pA increments. Histograms were fit with Gaussian distributions and the area-under-the-curve (AUC) for each was determined. Pₒ was calculated as \( \text{AUC}_{\text{channel-open}} / (\text{AUC}_{\text{channel-open}} + \text{AUC}_{\text{channel-closed}}) \). For estimation of channel open probability by stationary noise analysis, short recordings (~1 s) of macroscopic currents in K-INT/EDTA or K-INT/EDTA plus 5 mM ATP at −50 mV were used. For the mutants that are relatively insensitive to ATP inhibition, we reversed the polarity of currents observed in K-INT plus 10 mM BaCl₂ at ±50 mV and used that as the baseline. Currents were sampled at 50 kHz and filtered at 5 kHz. Mean current (i) and variance (σ²) in the absence of ATP were obtained by subtraction of the mean current and variance of the baseline. Single channel current (i) was assumed to be −3.6 pA at −50 mV in symmetrical K-INT solution (corresponding to single channel conductance of 72 picosiemens). Pₒ was then calculated using the equation: \( Pₒ = 1 - (\sigma²/(i × I)) \) (21, 22).

All data are presented as means ± S.E. Statistical analysis was performed using an independent two-population two-tailed Student’s t test, with \( p < 0.05 \) considered statistically significant.

**RESULTS**

**The R74W and E128K Mutations Reduce Channel Sensitivity to ATP**—Previously, we reported several CHI-associated SUR1 mutations that reduce surface expression of Kᵦ₅P channels could be rescued to the cell surface efficiently by sulfonylureas such as glibenclamide and tolbutamide (13, 15). These mutations are all in the TMD0 of SUR1 (amino acids 1–196) and include G7R, N24K, F27S, R74W, A116P, E128K, and V187D. The functional properties of rescued A116P and V187D mutant channels had been characterized in detail and shown to be normal (13). To test if the other five rescued TMD0-SUR1 mutant channels also have normal nucleotide sensitivities, we performed inside-out patch clamp recordings from COSm6 cells co-transfected with Kir6.2 and WT-SUR1 or mutant-SUR1. Cells were treated with 300 µM tolbutamide overnight followed by 2-h washout to increase mutant channel expression at the cell surface before recording. Note tolbutamide was used in this and subsequent functional experiments because of its low affinity and reversible binding to Kᵦ₅P channels to allow rapid washout. Of the five mutants, G7R, N24K, and F27S had WT-like or slightly increased ATP sensitivity, and either normal or reduced MgADP response that is commonly associated with CHI mutations (Fig. 1) (23). To our surprise, however, two of the mutants, R74W and E128K, exhibited significantly reduced ATP sensitivities (Fig. 2, A and B). The R74W mutation has been reported in two focal cases (disease causing a paternally derived mutation that was expressed due to loss of heterozygosity for the maternal allele) (24). The disease in both cases was similar to other focal cases: severe neonatal onset hypoglycemia, failed medical therapy with diazoxide, required surgery, and cured by local resection of the lesion (24). R74W was also reported as a compound heterozygous mutation with another mutation, R1215Q, in a patient with diffuse HI who failed medical therapy with diazoxide and required a near-total pancreatectomy (15, 25). The E128K mutation occurred in homozygous form in a child with diffuse hyperinsulinism, severe neonatal onset hypoglycemia, failed diazoxide therapy, and required near-total pancreatectomy for control of hypoglycemia (15).

The reduced ATP sensitivity-gating defect is typically linked to PNDM and has never been reported in CHI mutations. To ensure the altered ATP sensitivities were not due to the rescue procedure, we examined ATP inhibition in cells not pretreated with tolbutamide. Although expression levels of R74W and E128K mutants were very low, sufficient currents in a small fraction of transfected cells (identified by co-transfected GFP) were detected. ATP inhibition was as ineffective as in the tolbutamide-rescued channels (Fig. 2C), indicating the defect is intrinsic to the mutant channels.
Mechanisms of Reduced ATP Inhibition in R74W and E128K Channels—Several studies have shown that PNDM-causing SUR1 or Kir6.2 mutations can reduce channel ATP sensitivity by enhancing channel response to Mg-nucleotide stimulation (26, 27). In inside-out patches, MgADP stimulated channel activity in both R74W and E128K mutants (Fig. 3A); however, because the mutants were much less sensitive to nucleotide inhibition, it is difficult to directly compare their MgADP sensitivities to WT channels. We therefore tested the effect of MgADP on channel ATP sensitivity in the background of SUR1-NBD mutations such as G1479D and G1479R in NBD2 and K719M in NBD1, which are known to abolish channel response to MgADP stimulation (28). In the E128K/G1479R double mutants, ATP sensitivity was as reduced as the E128K mutant; the R74W/G1479D double mutant also showed significantly reduced ATP sensitivity, although to a lesser degree than the R74W single mutant (Fig. 3, B and C). We also combined E128K with NBD1 mutation K719M (E128K/K719M), and the resulting channels were as insensitive to ATP as E128K (not shown). These results indicate that, even if the mutations increased Mg-nucleotide stimulation, this effect alone could not explain the reduced ATP sensitivity in R74W and E128K.

Another mechanism that could lead to a reduced apparent channel ATP sensitivity is increased intrinsic P∞ (8, 29, 30). The recently identified PNDM-causing SUR1 F132L mutation is an example, so are many Kir6.2 mutations reported earlier (8, 29). To examine this possibility, we first measured the P∞ of the R74W and E128K channels expressed in COSm6 cells by single channel recording. These experiments show the mutant channels indeed have altered P∞ values for R74W and E128K (0.63 ± 0.06; Fig. 4B). Of note, 10 out of 11 patches of the E128K mutant had consistently lower P∞ with only one outlier showing a P∞ of 0.69 (Fig. 4B). The R74W mutant exhibited more variable P∞ values for R74W and E128K.

The ATP sensitivity observed in A and B is not due to the chemical chaperone rescue procedure. Scale bars: WT: 500 pA, 10 s; R74W and E128K: 200 pA, 10 s. B, ATP dose-response relationships. Parameters describing best-fit curves to the Hill equation (I50 = 1/[(ATP)/IC50]) and Hill coefficient (H), are shown. Error bars represent ± S.E. of 3–7 patches. Note the IC50 values obtained using the K-INT/EDTA solution are higher than those reported by others as inclusion of EDTA significantly reduces rundown by minimizing Mg2+-dependent breakdown of membrane phosphoinositides (20). C, representative traces of WT and mutant channels from cells that have not been pretreated with tolbutamide, indicating the decrease in ATP sensitivity observed in A and B is not due to the chemical chaperone rescue procedure. Scale bars: WT: 500 pA, 10 s; R74W and E128K: 200 pA, 10 s.
ranging from 0.01 to 0.88 (Fig. 4B). Two recordings representing both ends of the R74WPo spectrum are shown in Fig. 4A. We cannot rule out that in some patches more than one channel might be present; alternatively, the variation might arise from differences in the cellular state such as membrane phosphoinositide levels (31). As a control, we also analyzed the distribution of individual 
P
o values given parenthetically. Recordings were digitized at 50 kHz and filtered at 2 kHz. Scale bars: 5 pA and 2 s for the 10-s records, 5 pA and 200 μs for the expanded records. B, average 
P
o values ± S.E. are shown in the bar graph. The distribution of individual 
P
o values is displayed; the total number of patches analyzed is shown above each bar (*, p < 0.05, Student’s t test; error bars represent ± S.E.).

coupling between SUR1 and Kir6.2. Using the same single channel recording condition (K-INT/1 mM EDTA), we found the 
P
o of Kir6.2ΔC channels to be 0.04 ± 0.01 and the IC50 of ATP inhibition 379 ± 53 μM, with a Hill coefficient of 0.97 ± 0.12. These values represent the limit at which the mutations could affect channel 
P
o and ATP sensitivity if the mutations exert their effects by uncoupling SUR1 and Kir6.2.

Mutant Channel Biogenesis Defects and Correction by Sulfonylureas in Insulin-secreting Cells—The reduced ATP sensitivity of the R74W and E128K mutant channels predicts that, were the channels able to overcome their trafficking defect, they would be insensitive to metabolic stimuli and would cause β-cell dysfunction resembling neonatal diabetes. To test this, we first confirmed...
the trafficking and gating phenotypes of the mutants in INS-1 cells (clone 832/13) (18). INS-1 cells were infected with Kir6.2 and WT or mutant SUR1 adenoviruses. Western blot of SUR1. The complex-glycosylated mature form of SUR1 is indicated by the open arrow and the core-glycosylated immature form by the solid arrow. The upper band is undetectable in untreated R74W- and E128K-infected cells, indicating defective channel processing and trafficking. Sulfonylurea treatment, however, restores upper band expression. The same blot was probed for α-tubulin to confirm equal loading of protein samples. 

Surface immunostaining with FLAG-antibody of SUR1 showed that R74W and E128K mutant channels are only detected at the cell surface following tolbutamide treatment. K<sub>ATP</sub> surface expression in INS-1 cells was quantified using chemiluminescence assays. Under control conditions, R74W and E128K both express at 9% of WT. Sulfonylurea treatment greatly improves R74W and E128K expression to 90 and 80%, respectively. Error bars represent ± S.E. of three experiments. Islets isolated from rat pancreas were cultured for 48 h and then infected with recombinant adenoviruses. Exogenous K<sub>ATP</sub> biogenesis was tracked by Western blot of FLAG epitope. The upper band of SUR1 was only detected following 5 μM glibenclamide treatment. A nonspecific band in the E128K blot is shown to serve as a loading control.

![Figure 5](image)

**FIGURE 5.** R74W and E128K surface expression was rescued by sulfonylurea treatment in insulin-secreting cells. Processing and surface expression of K<sub>ATP</sub> channels was assessed in INS-1 cells infected with Kir6.2 and WT or mutant SUR1 adenoviruses. A, Western blot of SUR1. The complex-glycosylated mature form of SUR1 is indicated by the open arrow and the core-glycosylated immature form by the solid arrow. The upper band is undetectable in untreated R74W- and E128K-infected cells, indicating defective channel processing and trafficking. Sulfonylurea treatment, however, restores upper band expression. The same blot was probed for α-tubulin to confirm equal loading of protein samples. B, surface immunostaining with FLAG-antibody of SUR1 showed that R74W and E128K mutant channels are only detected at the cell surface following tolbutamide treatment. C, K<sub>ATP</sub> surface expression in INS-1 cells was quantified using chemiluminescence assays. Under control conditions, R74W and E128K both express at 9% of WT. Sulfonylurea treatment greatly improves R74W and E128K expression to 90 and 80%, respectively. Error bars represent ± S.E. of three experiments. D, islets isolated from rat pancreas were cultured for 48 h and then infected with recombinant adenoviruses. Exogenous K<sub>ATP</sub> biogenesis was tracked by Western blot of FLAG epitope. The upper band of SUR1 was only detected following 5 μM glibenclamide treatment. A nonspecific band in the E128K blot is shown to serve as a loading control.

The trafficking and rescue characteristics of R74W and E128K were also confirmed in rat islets. Islets were transduced with the K<sub>ATP</sub> subunit adenoviruses for 24 h followed with or without 5 μM glibenclamide treatment for 12 h. The sulfonylurea rescue effect was appraised via the presence of complex-glycosylated SUR1 protein on Western blots. As shown in Fig. 5D, the SUR1 mutants exhibited the same processing defects that were ameliorated by glibenclamide treatment.

**Expression of R74W or E128K Mutant Alters INS-1 Cell Responses to Glucose Stimulation**—Having established the sulfonylurea-dependent expression of mutant channels in INS-1 cells, we next determined if expression of the mutants at the cell surface alters membrane electrical properties. Channel activity was first assessed in intact cells using the on-cell voltage clamp (−50 mV membrane potential) configuration. INS-1 cells infected with the K<sub>ATP</sub> subunit recombinant viruses were exposed to 12 mM glucose medium for 3 h before recording. Surface expression of mutant channels was rescued by overnight treatment with 300 μM tolbutamide. Tolbutamide was removed during the high glucose stimulation to allow for washout prior to recording. In uninfected cells or cells expressing exogenous WT K<sub>ATP</sub> channel subunits, little channel activity was detected (Fig. 6A). No channel opening was observed in any of the uninfected cells (n = 19); the major-
SUR1 Mutations, Hyperinsulinism, and Diabetes

We then tested if expression of mutant channels altered the relationship between glucose and membrane potential using whole cell patch clamp recording. Resting membrane potential (RMP) at 12 mM glucose was determined following whole cell break-in in the current clamp mode (Fig. 7A). Our prior work has shown that membrane potential upon break-in before dialysis (initial RMP) is similar to that measured by perforated patch clamp recording (19). Cells were exposed to 200 μM diazoxide, a K<sub>ATP</sub> channel opener, after the membrane potential had reached steady state to confirm whole cell seal quality and observe the full extent of K<sub>ATP</sub>-dependent hyperpolarization (not shown). The average initial RMPs with and without tolbutamide pretreatment are shown in Fig. 7B. There is no significant difference between control cells and cells infected with WT subunits (the initial RMPs are −16.4 ± 3.5 mV and −20.7 ± 4.8 mV, respectively). In contrast, both R74W- and E128K-expressing cells receiving tolbutamide pretreatment were significantly more hyperpolarized at 12 mM glucose. The initial RMP was −33 ± 3.8 mV for R74W and −54 ± 4.0 mV for E128K. In some mutant virus-infected cells without tolbutamide pretreatment the initial RMP at 12 mM glucose also appeared more hyperpolarized than uninfected or WT-infected cells (see supplemental Fig. S2), although the averaged initial RMP was still more

ity of cells overexpressing exogenous WT channels also had no activity, but occasionally some single channel openings were detected (two of seven). In contrast, all cells infected with the R74W or E128K channel subunits and pretreated with tolbutamide had high on-cell activities (Fig. 6A). Consistent with the on-cell activity, upon patch excision into the inside-out patch clamp configuration, channels from cells infected with mutant subunits and pretreated with tolbutamide had significantly reduced ATP sensitivity compared with channels from WT-infected cells (Fig. 6B), further confirming surface expression of the mutant channels.

Cells that were infected with R74W or E128K but not rescued by tolbutamide were also tested; a small fraction of each mutant displayed some on-cell channel activity but less than that observed in tolbutamide-treated cells and decreased channel ATP sensitivity upon patch excision (supplemental Fig. S1), indicating some mutant channels were able to traffic to the plasma membrane without pharmacologic chaperone. We suggest that this may be a consequence of heterologous overexpression. These results are in line with our hypothesis that mutant channels are hyperactive under high glucose conditions.

We also tested if expression of mutant channels altered the relationship between glucose and membrane potential using whole cell patch clamp recording. Resting membrane potential (RMP) at 12 mM glucose was determined following whole cell break-in in the current clamp mode (Fig. 7A). Our prior work has shown that membrane potential upon break-in before dialysis (initial RMP) is similar to that measured by perforated patch clamp recording (19). Cells were exposed to 200 μM diazoxide, a K<sub>ATP</sub> channel opener, after the membrane potential had reached steady state to confirm whole cell seal quality and observe the full extent of K<sub>ATP</sub>-dependent hyperpolarization (not shown). The average initial RMPs with and without tolbutamide pretreatment are shown in Fig. 7B. There is no significant difference between control cells and cells infected with WT subunits (the initial RMPs are −16.4 ± 3.5 mV and −20.7 ± 4.8 mV, respectively). In contrast, both R74W- and E128K-expressing cells receiving tolbutamide pretreatment were significantly more hyperpolarized at 12 mM glucose. The initial RMP was −33 ± 3.8 mV for R74W and −54 ± 4.0 mV for E128K. In some mutant virus-infected cells without tolbutamide pretreatment the initial RMP at 12 mM glucose also appeared more hyperpolarized than uninfected or WT-infected cells (see supplemental Fig. S2), although the averaged initial RMP was still more

A

B

C

FIGURE 7. R74W or E128K expression at the plasma membrane results in a diabetes phenotype. INS-1 cells were co-infected with the K<sub>ATP</sub> subunit-encoding adenoviruses followed by pretreatment with 300 μM tolbutamide and washout to rescue surface expression where indicated. A and B, initial and post-break-in steady-state membrane potentials following 12 mM glucose stimulation were determined by whole cell current clamp recordings. Representative traces are shown in A. Scale bars represent 10 s of recording, and the downward arrow specifies the time of break-in. The initial spike is an artifact going from on-cell to whole cell mode. The average membrane potential values are shown in B. Each bar represents the mean ± S.E. of 11–30 cells. *p < 0.05; †, p < 0.01, Student’s t test. C, insulin secretion at basal (3 mM) and 12 mM glucose in uninfected controls and WT-, R74W-, or E128K-infected INS-1 cells. R74W- and E128K-infected cells pretreated with 300 μM tolbutamide for 4 h to rescue surface expression had significantly less insulin secretion relative to control or WT-infected cells. In R74W- or E128K-infected cells without tolbutamide rescue, insulin secretion was also reduced likely due to some leak expression of the mutants, although the extent of reduction was less than tolbutamide-rescued cells. *, p < 0.05; †, p < 0.01, Student’s t test. Each bar represents the mean ± S.E. of three to five experiments.

Lasty, we determined if rescue of the R74W or E128K mutant channels to the cell surface would cause defective insulin secretion in response to glucose stimulation. Insulin release during a 2-h static incubation in basal (3 mM) or high (12 mM) glucose was compared for the same experimental groups tested in the above electrophysiology studies. Because prolonged tolbutamide pretreatment to rescue mutant channel surface expression may desensitize cells to subsequent glucose stimulation by affecting their insulin secretory capacity (36, 37) and confound data interpretation, we optimized the tolbutamide pretreatment such that no reduction in subsequent glucose-stimulated insulin secretion was observed in uninfected control
SUR1 Mutations, Hyperinsulinism, and Diabetes

cells. We found that pretreating INS-1 cells with tolbutamide for 4 h rescued mutant surface expression without affecting insulin secretory capacity. Using this experimental paradigm, we observed that secretion in 12 mM glucose was significantly reduced in tolbutamide- rescued, mutant-infected cells compared with uninfected or WT-infected cells (Fig. 7C). In agreement with the electrophysiological data, INS-1 cells infected with mutant channel viruses but not treated with tolbutamide also caused a somewhat reduced insulin secretion response to high glucose, again due to tolbutamide-independent leak expression of mutant channels at the cell surface. These data led us to conclude that rescue of the CHI-causing R74W or E128K mutant K_{ATP} channels by sulfonylureas inverses the β-cell dysfunction phenotype to diabetic.

**DISCUSSION**

Studies of K_{ATP} channel mutations from patients with hyperinsulinism or diabetes have led to the general view that mutations from hyperinsulenic patients cause reduced channel function owing to inability of the mutant to express at the cell surface and/or to open during hypoglycemia, whereas those from neonatal diabetes patients cause enhanced channel function by rendering the channel insensitive to the increased ATP/ADP ratio during glucose stimulation (2). In this work, we present the novel finding of two SUR1 mutations that cause severe hyperinsulinism in patients by preventing channel expression at the cell surface, surprisingly, also render the channel insensitive to ATP inhibition. The diabetes-inducing gating defects were clearly revealed after the mutant channels were rescued to the cell surface by their pharmacological chaperones sulfonylureas. As predicted by the reduced ATP sensitivity-gating defects, expression of mutant channels at the surface of INS-1 cells resulted in insensitivity of the cell to glucose stimulation consistent with a diabetes-like phenotype.

**Mechanisms of Reduced ATP Sensitivities in the Mutant Channels**—Our results indicate that the decreased ATP sensitivities of R74W and E128K are not due to enhanced MgADP stimulation, at least alone, because elimination of channel MgADP response by mutations in the nucleotide binding folds did not restore their ATP sensitivity to the level of WT channels (Fig. 3). Furthermore, we found that, rather than increasing channel intrinsic P_o, the R74W and E128K mutations significantly lowered the average intrinsic P_o (Fig. 4). These properties are unlike the previously reported ATP-insensitive mutants and place the R74W and E128K mutants in a distinct category in terms of the underlying mechanisms for loss of ATP sensitivity. Studies by others have indicated that TMD0 physiologically couples to Kir6.2 to modulate channel gating and facilitate surface expression (9, 10). However, the chemical interactions between TMD0 and Kir6.2 critical for such modulation have not been defined. That the E128K mutant has poor surface expression, lower P_o, and reduced ATP sensitivity closer to those seen in Kir6.2AC channels suggests the mutation likely disrupts the functional coupling between TMD0-SUR1 and Kir6.2. The R74W mutation might also disrupt functional interactions between TMD0-SUR1 and Kir6.2; however, this disruption would be predicted to be less pronounced as the mutation only caused mild reduction in ATP sensitivity and P_o. It is possible that Arg-74 and Glu-128 form interactions with residues in Kir6.2 that are critical for the cross-talk between the two subunits. Interestingly, comparison between channels formed by Kir6.2AC only and channels formed by co-expression of TMD0-SUR1 with Kir6.2AC showed that TMD0-SUR1 further decreases the ATP sensitivity of Kir6.2AC channels by ~6-fold (9, 30). Because TMD0-SUR1 decreases whereas full-length SUR1 increases the ATP sensitivity of Kir6.2AC, the hypersensitizing effect of full-length SUR1 on channel ATP sensitivity must require domains other than TMD0 (9). This, however, does not exclude a role of TMD0 in mediating the hypersensitizing effect of full-length SUR1 on channel ATP sensitivity. In fact, the reduced ATP sensitivities observed in the R74W and E128K mutants indicate TMD0 is necessary for normal channel ATP sensitivity. A possible scenario is that TMD0 serves as a conduit between other SUR1 domains and Kir6.2 to transduce this hypersensitization.

If R74W and E128K cause functional uncoupling between TMD0-SUR1 and Kir6.2, one might ask if the mutations also result in reduced physical association between the two subunits. Several SUR1-TMD0 mutations have been reported to reduce physical association between TMD0 and Kir6.2 in co-immunoprecipitation experiments, including CHI-causing A116P and V187D mutations and PNDM-causing F132L mutation (10, 30). The former two do not affect the gating properties of the channel (13), whereas the F132L mutation reduces ATP sensitivity by increasing channel intrinsic P_o (30). These studies suggest that physical association, as assessed by co-immunoprecipitation, does not necessarily correlate with the functional relationship between TMD0 and Kir6.2. It would not be surprising if R74W and E128K do not affect the extent of co-immunoprecipitation between SUR1 and Kir6.2, because there are likely multiple chemical interactions retained (such as those mediated by Ala-116, Val-187, and Phe-132) to allow association of the two subunits.

**The Interplay between Channel Expression and Gating in Determining Disease Phenotype**—Our study highlights the importance of mutant channel expression level at the cell surface in determining disease phenotype. R74W and E128K, like all other neonatal diabetes-causing mutations, render K_{ATP} channels less sensitive to ATP inhibition during glucose stimulation, and yet they were identified in patients with severe hyperinsulinism, because they also prevent channels from being expressed at the cell membrane. When brought to the INS-1 cell surface by chemical chaperones, the effects of their reduced ATP sensitivity on short circuiting cell response to glucose stimulation became apparent, with the extent of defects correlated with the extent of ATP insensitivities observed in the mutants (Fig. 7). Interestingly, our recent study comparing dominant versus recessive forms of CHI associated with K_{ATP} channel mutations found that, whereas recessive CHI is associated with mutations that often prevent channel expression at the cell surface, dominant CHI is associated with heterozygous mutations that do not compromise channel surface expression but do impair the ability of channels to open in low glucose (38). The R74W was identified in one patient with diffuse disease who also carries an R1215Q mutation that reduces channel sensitivity to MgADP and two patients with focal disease (23–25),
and E128K was identified as a disease-causing homozygous mutation in a patient with diffuse disease (15). In β-cells of these patients, the mutant channel expression level at the surface is likely so low that their gating defects are completely hidden. This is in contrast to dominant heterozygous CHI-causing mutations and PNDM-causing heterozygous mutations in which sufficient mutant subunits co-assemble with WT subunits in the octameric channel complex and traffic to the cell surface to manifest their gating defects (17).

**Sulfonylureas, Insulin Secretion Diseases, and Therapy**—The therapeutic utility of sulfonylureas has recently been extended beyond type II diabetes to some patients with PNDM or developmental delay, epilepsy, and neonatal diabetes syndrome caused by $K_{\text{ATP}}$ channel mutations (2, 39). Moreover, the finding that sulfonylureas could enhance $K_{\text{ATP}}$ channel biogenesis and overcome channel trafficking defects caused by CHI-associated TMD0-SUR1 mutations raises the possibility that these drugs might also have future application to the treatment of some cases of CHI (13, 15). The study we presented here clearly illustrates the complex actions of sulfonylureas and the need to carefully weigh their effects on channel expression and channel gating when treating patients. In this regard, it is important to note that we have found the neonatal diabetes-causing mutation F132L also significantly reduces channel expression at the cell surface (57.05 ± 1.75% of WT; $n = 3$), and that sulfonylureas restore mutant channel surface expression to the same level as WT (109.2 ± 7.05; $n = 3$). Detailed functional analyses of individual disease mutations will be important to develop genotype-specific and mechanism-based therapeutic strategies.

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