Sulfonylureas Correct Trafficking Defects of Disease-causing ATP-sensitive Potassium Channels by Binding to the Channel Complex

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ATP-sensitive potassium (K$_{ATP}$) channels mediate glucose-induced insulin secretion by coupling metabolic signals to β-cell membrane potential and the secretory machinery. Reduced K$_{ATP}$ channel expression caused by mutations in the channel proteins: sulfonylurea receptor 1 (SUR1) and Kir6.2, results in loss of channel function as seen in congenital hyperinsulinism. Previously, we reported that sulfonylureas, oral hypoglycemic drugs widely used to treat type II diabetes, correct the endoplasmic reticulum to the plasma membrane trafficking defect caused by two SUR1 mutations, A116P and V187D. In this study, we investigated the mechanism by which sulfonylureas rescue these mutants. We found that glinides, another class of SUR-binding hypoglycemic drugs, also markedly increased surface expression of the trafficking mutants. Attenuating or abolishing the ability of mutant SUR1 to bind sulfonylureas or glinides by the following mutations: Y230A, S1238Y, or both, accordingly diminished the rescuing effects of the drugs. Interestingly, rescue of the trafficking defects requires mutant SUR1 to be co-expressed with Kir6.2, suggesting that the channel complex, rather than SUR1 alone, is the drug target. Observations that sulfonylureas also reverse trafficking defects caused by neonatal diabetes-associated Kir6.2 mutations in a way that is dependent on intact sulfonylurea binding sites in SUR1 further support this notion. Our results provide insight into the mechanistic and structural basis on which sulfonylureas rescue K$_{ATP}$ channel surface expression defects caused by channel mutations.

Regulation of insulin secretion by blood glucose relies on expression of functional ATP-sensitive potassium (K$_{ATP}$) channels at the β-cell membrane. The β-cell K$_{ATP}$ channel is an octameric protein complex comprising four pore-forming Kir6.2 subunits and four regulatory sulfonylurea receptor 1 (SUR1)$^2$ subunits (1–3). Channel activity is determined by the interplay between both channel subunits and intracellular ATP and ADP: binding of ATP to the Kir6.2 subunit inhibits channel activity, whereas binding of Mg$^{2+}$-complexed ATP or ADP to the SUR1 subunit stimulates channel activity (4–6). In this way, channel activity serves as a reporter of intracellular ATP and ADP concentrations during glucose metabolism to control β-cell excitability, hence insulin secretion. Dysfunction of β-cell K$_{ATP}$ channels because of mutations in the channel subunits Kir6.2 or SUR1 underlies congenital insulin secretion disorders (4, 5). Whereas mutations causing loss of channel function lead to excessive insulin secretion and hypoglycemia as seen in patients with congenital hyperinsulinism (CHI), those causing gain of channel function lead to insufficient insulin secretion and permanent neonatal diabetes mellitus (PNDM).

In congenital hyperinsulinism, two prominent mechanisms accounting for loss of channel function are loss of channel expression at the cell surface and loss of channel sensitivity to stimulation by MgADP (7). In contrast, gain of channel function associated with heterozygous mutations in Kir6.2 or SUR1 in neonatal diabetes is thought to result from reduced channel inhibition at physiological concentrations of MgATP (8, 9). Interestingly, we recently showed that some PNMD-causing Kir6.2 mutations also reduce the efficiency of channel expression at the cell surface, although the loss of expression effect is masked by the ATP gating defect, resulting in a net gain of channel function, thereby the diabetes phenotype (10).

Sulfonylureas such as tolbutamide and glibenclamide are oral hypoglycemic drugs commonly used for treating type II diabetes; they do so by binding to the channel, primarily to the SUR1 subunit, and inhibiting channel activity (4, 11, 12). Glibenclamide differs from tolbutamide in that it has an additional benzamido moiety attached to the sulfonylurea moiety. Glibenclamide binds to SUR1 with ∼1,000-fold higher affinity than tolbutamide, and blocks channel activity at a much lower concentration with the block being irreversible (11). Another class of compounds known as glinides, for example, meglitinide and repaglinide, which are structurally more related to the non-sulfonylurea half of glibenclamide, also bind SUR1 to inhibit channel activity (11, 13, 14). The binding pocket of glibenclamide is therefore proposed to comprise two sites: site A, which binds ligands with the short chain sulfonylurea moiety like tolbutamide and site B, which binds the non-sulfonylurea half of glibenclamide, as well as glinides such as repaglinide, as

bovine serum albumin; PBS, phosphate-buffered saline; ER, endoplasmic reticulum; WT, wild type.

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

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2 The abbreviations used are: SUR1, sulfonylurea receptor 1; CHI, congenital hyperinsulinism; PNMD, permanent neonatal diabetes mellitus; BSA,
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A

![Topology model of SUR1](image)

B

![Pharmacophore model for sulfonylureas](image)

**FIGURE 1.** A, topology model of SUR1 showing the three transmembrane domains, the location of the Y230A mutation, and the location of the S1238Y and A116P mutations that have been proposed to disrupt the A- and B- sulfonylurea binding sites (as shown in B), respectively. B, pharmacophore model for sulfonylureas (modified from Grell et al. (30)). The chemical structures of tolbutamide, glibenclamide, and repaglinide are also shown.

shown in Fig. 1 (13, 15). Sites A and B are thought to overlap to accommodate the negative charge and the central phenyl ring present in both sulfonylureas and glinides (Fig. 1) (15).

SUR1 belongs to the ATP-binding cassette (ABC) transporter protein family (16); it has three transmembrane domains, TMD0, TMD1, and TMD2 each containing 5, 6, and 6 transmembrane segments, and two intracellular nucleotide-binding domains (Fig. 1) (17, 18). Structure-function and binding studies using recombinant channel proteins reveal two SUR1 regions that are critical for binding (14, 16, 19−22). The first region, likely representing the A site, involves loop linking transmembrane segments 15 and 16 of TMD2. Mutation of a single serine residue (Ser1237 of rat SUR1) in this region abolishes $[^{3}H]$glibenclamide binding to SUR1 and in electrophysiological experiments, the same mutation abolishes channel inhibition by tolbutamide and renders channel inhibition by glibenclamide readily reversible (20). The second region involves the cytoplasmic loop between TMD0 and TMD1 (referred to as L0 by Bryan et al. (14)) where the B site may be located. Deletion of this cytoplasmic loop leads to loss of $[^{3}H]$glibenclamide binding in recombinant SUR1 expressed in insect cells (21) and mutation of tyrosine 230 in L0 to alanine (Y230A) abolishes photoaffinity labeling of SUR1 by $[^{125}]$azido-glibenclamide (14).

Although the effect of sulfonylureas on $K_{ATP}$ channel activity has been known for a long time, their effect on $K_{ATP}$ channel expression/trafficking was only recently appreciated. We have previously reported that sulfonylureas rescue surface expression defects of $K_{ATP}$ channels caused by two CHI-associated SUR1 mutations, A116P and V187D (23). More recently, we found that several Kir6.2 mutations identified in PNDM also reduce channel surface expression and that the reduced surface expression is improved upon sulfonylurea treatment (10). In this work, we investigated the mechanism by which sulfonylureas correct the channel surface expression defects caused by the A116P or V187D mutations and by the PNDM-associated Kir6.2 mutations. Our results indicate that both the sulfonylurea and the glinide moieties contribute to the rescue effect and show that SUR1 mutations known to interfere with drug binding also interfere with the ability of these drugs to rescue the mutant channel surface expression defect. Interestingly and somewhat unexpectedly, we found that Kir6.2 is required for sulfonylureas to rescue the A116P and V187D mutant SUR1 at the cell surface. Conversely, sulfonylurea binding at SUR1 is necessary for the drug to improve surface expression of PNDM mutant channels harboring Kir6.2 mutations. These results suggest that sulfonylureas exert chaperoning effects on the SUR1-Kir6.2 complex rather than mutant SUR1 or Kir6.2 alone to correct $K_{ATP}$ channel surface expression defects.

**MATERIALS AND METHODS**

**Molecular Biology**—FLAG epitope (DYKDDDDK) was inserted at the N terminus of the hamster SUR1 cDNA by sequential over-lap extension PCR (referred to as fSUR1), as described previously (24). Point mutations of SUR1 were introduced into hamster SUR1 cDNA in the pECE plasmid using the QuikChange site-directed mutagenesis kit (Stratagene). The FLAG epitope tag and mutations were confirmed by DNA sequencing. Rat Kir6.2 cDNA is in the pcDNAI vector. Mutant clones from multiple PCR were analyzed in all experiments to avoid false results caused by undesired mutations introduced by PCR.

**Immunofluorescence Staining**—COSm6 cells were plated in 6-well tissue culture plates, transfected with 0.6 μg of fSUR1 and 0.4 μg of Kir6.2 per well using FuGENE 6 (Roche) according to the manufacturer's directions. Transfected cells in each well were equally split and replated onto three coverslips 24 h post-transfection. Two coverslips were used for surface staining and one was used for total staining of fSUR1. For surface staining, cells were pretreated with or without 5 μM glibenclamide (diluted in culture medium from 10 mM stock dissolved in dimethyl sulfoxide (Me2SO)) 24 h prior to the staining. Staining was carried out as described previously (23). Briefly, cells were incubated with anti-FLAG M2 mouse monoclonal antibody (Sigma, diluted to 10 μg/ml in Opti-MEM containing 0.1% BSA) for 1 h at 4 °C, washed with ice-cold PBS, then incubated with Cy3-conjugated donkey anti-mouse secondary antibodies (Jackson) for 30 min at 4 °C. After three 5-min washes in ice-cold PBS, cells were fixed with 4% paraformaldehyde, and viewed using an Olympus Fluoview confocal microscope. For total cellular staining of fSUR1, cells were fixed with cold
(-20°C) methanol for 5 min. Fixed cells were incubated with the anti-FLAG M2 monoclonal antibody (10 µg/ml PBS containing 0.1% BSA) at room temperature for 1 h, washed in PBS, incubated with Cy3-conjugated donkey anti-mouse secondary antibodies for 30 min at room temperature, and washed again in PBS before imaging.

**Immunoblotting**—COSm6 cells were plated in 35-mm dishes and transfected with K\textsubscript{ATP} channel subunits using FuGENE 6 as previously described (23). Cells were lysed 48–72 h post-transfection in a buffer containing 20 mM Hepes (pH 7.0), 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, and Complete
\textsuperscript{TM} protease inhibitors (Roche). Proteins in cell lysates were separated by SDS-PAGE (8%), transferred to nitrocellulose membrane, incubated with the M2 anti-FLAG antibody (Sigma) followed by horseradish peroxidase-conjugated anti-mouse secondary antibodies (Amersham Biosciences), and visualized by chemiluminescence (Super Signal West Femto; Pierce).

**Chemiluminescence Assay**—Transfection of COSm6 cells was carried out as described above. Drug treatment was initiated 32–40 h post-transfection and lasted for 24 h. Cells were then processed for chemiluminescence assays as described previously (25). Briefly, cells were fixed with 2% paraformaldehyde for 30 min at 4°C, preblocked in PBS, 0.1% BSA for 30 min, incubated in M2 anti-FLAG antibody (10 µg/ml) for 1 h, washed four times for 30 min in PBS, 0.1% BSA, incubated in horseradish peroxidase-conjugated anti-mouse antibody (Jackson, 1:1000 dilution) for 20 min, and washed again four times for 30 min in PBS, 0.1% BSA. Chemiluminescence of each dish was quantified in a TD-20/20 luminometer (Turner Designs) following a 5-s incubation in Power Signal Elisa Femto luminol solution (Pierce). All steps after fixation were carried out at room temperature.

**Electrophysiology**—COSm6 cells were transfected using FuGENE 6 and plated onto coverslips. The cDNA for the green fluorescent protein was cotransfected with SUR1 and Kir6.2 to facilitate identification of positively transfected cells. Patch clamp recordings were made 36–72 h post-transfection. All experiments were performed at room temperature as previously described (24). Micropipettes were pulled from non-heparinized Kimble glass (Fisher Scientific) on a horizontal puller (Sutter Instrument, Co., Novato, CA). Electrode resistance was typically 1.4–1.6 MΩ when filled with K-INT solution (below). Inside-out patches were voltage-clamped with an Axopatch 1D amplifier (Axon Inc., Foster City, CA). The standard bath (intracellular) and pipette (extracellular) solutions (K-INT) had the following composition: 140 mM KCl, 10 mM K-HEPES, 1 mM K-EGTA (pH 7.3). ATP was added as the potassium salt. Tolbutamide and glibenclamide were dissolved in Me\textsubscript{2}SO at 300 or 10 mM, respectively, and diluted further in K-INT. Control experiments (see supplemental Fig. S1) confirm that Me\textsubscript{2}SO at the final concentrations used does not affect K\textsubscript{ATP} channel activity (26). All currents were measured at a membrane potential of -50 mV (pipette voltage = +50 mV), and inward currents shown as upward deflections. Data were analyzed using pCLAMP8 software (Axon Instrument). Off-line analysis was performed using Origin 6.1 and Microsoft Excel programs.

**Data Analysis**—Data were presented as mean ± S.E. Statistical analysis was performed using independent two-popula-

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**RESULTS**

**TMD0 of SUR1 Does Not Confer the Rescue Effect of Sulfonylureas on Mutant Expression**—Many CHI-causing SUR1 mutations have been reported to reduce surface expression of K\textsubscript{ATP} channels by causing ER retention of the mutant channel (they are referred to as trafficking mutations hereafter for their inability to traffic normally to the plasma membrane) (23–25, 27). Of these, two mutations, A116P and V187D, were rescued by the pharmacological agent sulfonylurea (23). We hypothesize that sulfonylurea may rescue these mutants by binding to the mutant protein and acting as chemical chaperones to facilitate channel biogenesis and trafficking. Because both A116P and V187D are located in TMD0, we first tested if sulfonylureas bind directly to TMD0 to facilitate mutant protein biogenesis and trafficking, even though TMD0 has not been implicated in sulfonylurea binding in prior studies (21). We compared surface expression of recombinant SUR1-TMD0 (amino acids 1–197) containing either the A116P or the V187D mutation in the presence or absence of 5 µM glibenclamide. Studies by others have shown that co-expression of SUR1-TMD0 with Kir6.2 C-terminal deletion mutant Kir6.2ΔC25, which lacks the -RKR- ER retention motif, reconstitutes “mini” K\textsubscript{ATP} channels at the cell surface (27, 28). Consistently, we observed cell surface expression of SUR1-TMD0 (with a FLAG epitope tag placed at the N terminus; referred to as fSUR1-TMD0) in COSm6 cells transiently transfected with the construct and Kir6.2ΔC25 using chemiluminescence assays (Fig. 2). By con-

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**Sulfonylureas and K\textsubscript{ATP} Channel Trafficking**

![Image](https://example.com/sulfonylureas_atp_channel_trafficking.png)

**FIGURE 2.** The first transmembrane domain of SUR1 (TMD0) does not confer the sulfonylurea rescue effect on the A116P or V187D mutations. A, schematic showing the fSUR1-TMD0 (amino acids 1–197) and the Kir6.2ΔC25 constructs used in experiments shown in B. B, surface expression of fSUR1-TMD0 harboring mutations A116P or V187D in cells treated with or without glibenclamide (5 µM for 24 h). All cells were cotransfected with Kir6.2ΔC25. Surface expression level of WT fSUR1-TMD0 in untreated cells was taken as 100%. Each bar represents the mean ± S.E. of three to four independent experiments.
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**A**

- **Surface**
  - WT
  - Y230A
  - S1238Y
  - Y230A/S1238Y

- **Total**

**B**

- **Surface**
  - A116P
  - A116P/Y230A
  - A116P/S1238Y
  - A116P/Y230A/S1238Y

- **Total**

- **Surface + 5μM Glib**

**C**

- **Surface**
  - V187D
  - V187D/Y230A
  - V187D/S1238Y
  - V187D/Y230A/S1238Y

- **Total**

- **Surface + 5μM Glib**
trast, when the A116P or V187D mutations were introduced into fSUR1-TMD0, surface expression was greatly reduced (by >70%), even though the total mutant recombinant proteins were abundantly expressed as assessed by Western blots and immunofluorescent staining of permeabilized cells (not shown). Treating cells with 5 μM glibenclamide did not improve surface expression of the mutant fSUR1-TMD0. These results demonstrate that the TMD0 domain of SUR1 alone is insufficient to confer the sulfonylurea rescue effect.

Restoration of A116P- and V187D-mutant Channel Expression by Sulfonylureas Is Dependent on Intact Sulfonylurea Binding Sites in SUR1—Several studies indicate that the high affinity tolbutamide binding site in SUR1 resides in transmembrane segments 13–16 (19, 20). Specifically, mutation of a serine residue in this region (Ser1237 of rat SUR1) to tyrosine abolishes the high affinity block of channel activity by tolbutamide (20). The same mutation also reduces [3H]glibenclamide binding to N1 and renders channel inhibition by glibenclamide readily reversible (20). If sulfonylureas rescue the A116P and V187D trafficking mutants by binding to the channel protein, then introducing the S1237Y mutation should also reduce or abolish the ability of sulfonylureas to correct the trafficking defect. We made the equivalent sulfonylurea binding site mutation (S1238Y) in hamster SUR1 (16) and examined how it affects the response of the A116P- or V187D-mutant channels to sulfonylureas. Initial assessment by immunofluorescent staining indicates that the S1238Y mutation by itself does not affect fSUR1 surface expression when coexpressed with Kir6.2; however, when combined with the A116P or V187D mutation, it indeed reduced or prevented the ability of glibenclamide to rescue the surface expression defect caused by the A116P and V187D mutations (Fig. 3). Staining of permeabilized cells transfected with the various mutants revealed similar total mutant protein expression levels. Consistent results were obtained using Western blot analysis. A representative blot of A116P fSUR1 in the WT or S1238Y background from cells treated with or without 5 μM glibenclamide for 24 h is shown in Fig. 4A. We next sought to quantify mutant channel expression at the cell surface in the presence or absence of sulfonylurea treatment. Although K\textsubscript{ATP} current density provides a measure for surface expression of the SUR1-Kir6.2 octameric channel complex, it is not suitable for assessing the effect of glibenclamide on surface channel expression because glibenclamide, even after extensive washes, remains bound to channels rescued to the cell surface and inhibits channel activity (see supplemental Figs. S2 and S3) (23). The inhibition by prebound glibenclamide in electrophysiological experiments causes underestimation of channel expression. We therefore resorted to chemiluminescence assays that have been used extensively in prior studies to quantify surface channel expression (23, 25, 26, 29). As shown in Fig. 4B, channels containing the S1238Y-SUR1 mutation alone had surface expression levels comparable with that of the WT channels (see also Fig. 3 and supplemental Fig. S3A). However, when combined with the A116P- or V187D-SUR1 trafficking mutations, S1238Y completely abolished the rescue effect of tolbutamide at 300 (Fig. 4B) and 600 μM (not shown). The S1238Y mutation also significantly reduced the potency of glibenclamide rescue (Fig. 4C). In the WT background (no sulfonylurea binding mutation), 24-h treatment with 1 μM glibenclamide increased surface expression of the A116P mutant from 3.1 ± 0.7 to 34.5 ± 7.2% and the V187D mutant from 12.8 ± 1.6 to 49.2 ± 6.8% of WT channels. But in the S1238Y background, the same treatment only slightly increased surface expression of the A116P and V187D mutants, from 0.7 ± 1.0 to 6.6 ± 2.1% and 9.8 ± 1.4 to 15.6 ± 2.3% of WT, respectively. Increasing the concentration of glibenclamide to 5 μM led to a much greater effect on surface expression of the mutants (to 21.8 and 23.9% of WT for A116P and V187D, respectively; Fig. 4C). Thus, whereas tolbutamide response was abolished by the S1238Y mutation, the glibenclamide response appears to be only partially affected. Note that the Me\textsubscript{6}SO present in the sulfonylurea stock solutions had no effect on surface expression of the channel (data not shown).

Unlike tolbutamide, glibenclamide has a benzamido (meglitinide) moiety in addition to the sulfonylurea moiety and is thought to interact with two sites in SUR1 (see Fig. 1) (15, 21, 30). The observation that S1238Y did not completely abolish the ability of glibenclamide to rescue the trafficking mutants suggests that interaction of the benzamido moiety with an additional site, likely the B site, also contributes to the rescuing effect. Bryan et al. (14) have shown that mutation of Tyr\textsuperscript{230} located in the intracellular loop between TMD0 and TMD1 to alanine (Y230A) results in loss of [125I]jazidoglibenclamide photofinity labeling, suggesting that the benzamido group lies in close proximity to Tyr\textsuperscript{230} during binding, although it is possible that Y230A abolishes binding indirectly by affecting a distant site. We therefore tested whether this mutation also interferes with the ability of sulfonylureas to rescue the A116P and V187D trafficking mutants. Indeed, the Y230A mutation reduced the rescue effect of glibenclamide, as assessed by immunostaining (Fig. 3) as well as chemiluminescence assays (Fig. 5A). We then asked whether this reduction is attributable to loss of binding of

FIGURE 3. Impact of the sulfonylurea binding mutations on the effectiveness of glibenclamide to rescue K\textsubscript{ATP} channel trafficking defects in the presence of Kir6.2. Using immunocytochemistry analysis. A, top panel, surface staining of COS6 cells transiently transfected with Kir6.2 and WT-, Y230A-, S1238Y-, or Y230A/S1238Y-fSUR1, using the M2 anti-FLAG mouse monoclonal antibodies followed by Cy3-conjugated anti-mouse secondary antibody. Staining was performed in living cells at 4 °C, cells were then fixed with 4% paraformaldehyde and viewed by confocal microscopy (Olympus Fluoview 300). Bottom panel, total cellular expression of WT or mutant fSUR1. Cells cotransfected with Kir6.2 and the various fSUR1 constructs were fixed and permeabilized with methanol and stained for the FLAG epitope using the M2 anti-FLAG mouse monoclonal antibodies followed by Cy3-conjugated anti-mouse secondary antibody. All sulfonylurea binding mutants showed surface expression as well as total staining comparable with WT. B, addition of the A116P mutation in WT or the various sulfonylurea binding mutation backgrounds abolished surface staining (top panel), although the mutant proteins were detected inside the cell (middle panel). Note the mutant proteins detected inside the cell exhibited perinuclear staining patterns indicative of ER retention. Treatment of cells with 5 μM glibenclamide significantly increased surface expression of the A116P mutant as reported previously. The Y230A or S1238Y mutations reduced the ability of glibenclamide to restore surface expression of the A116P mutant and simultaneous mutation of Y230A and S1238Y completely abolished the ability of glibenclamide to rescue A116P to the cell surface. C, same as B except that the V187D trafficking mutation was introduced into WT or sulfonylurea binding mutation fSUR1 background. Note as previously reported, the V187D mutation showed slightly higher surface expression compared with the A116P mutation, with faint surface staining barely visible (23).
the benzamido moiety to SUR1. Accordingly, we examined how repaglinide, a glinide that contains the benzamido moiety but not the sulfonylurea moiety, affects surface expression of A116P and V187D channels in the presence or absence of the Y230A mutation. Fig. 5B shows that repaglinide at 10 μM effectively rescued surface expression of the A116P and V187D mutants, whereas the Tyr230 mutation abolished this rescue effect; in contrast, the S1238Y mutation had little effect on the ability of repaglinide to rescue the trafficking mutants (not shown). Surprisingly, we found that the Y230A mutation also renders tolbutamide unable to rescue the A116P and V187D trafficking mutants (Fig. 5C), suggesting a role of Tyr230 in tolbutamide binding or in conferring tolbutamide sensitivity toward trafficking rescue through an allosteric effect. Finally, introducing the Y230A and S1238Y mutations simultaneously completely abolished the rescue effect by tolbutamide, repaglinide, or glibenclamide (Figs. 3 and 6). Together, the above results demonstrate that sulfonylureas as well as glinides rescue

FIGURE 4. Effect of the S1238Y mutation on the ability of sulfonylureas to rescue K<sub>ATP</sub> channel trafficking defects in the presence of Kir6.2, analysis by immunoblotting and chemiluminescence assays. A, Western blot analysis of fSUR1. In cells expressing Kir6.2 and WT-fSUR1, two bands were observed: the lower core glycosylated band, or the immature band (open arrow) and the upper complex glycosylated band, or the mature band (solid arrow). For the A116P-fSUR1, however, only the immature band was detected in untreated cells (A116P); upon treatment with 1 μM glibenclamide for 24 h (A116P + Glib), the upper A116P-fSUR1 band became apparent, indicating rescue of the mutant protein out of the ER. When the S1238Y mutation was introduced into A116P-fSUR1 (A116P/S1238Y), the same glibenclamide treatment was less effective in promoting expression of the upper band (A116P/S1238Y + Glib). B, quantification of channel expression at the cell surface by chemiluminescence assays. COS<sub>6</sub> cells were co-transfected with Kir6.2 and one of the fSUR1 constructs indicated below the x-axis, and treated with or without 300 μM tolbutamide (Tolb) for 24 h prior to the assay. Surface expression of tolbutamide-treated cells was significantly higher than untreated for A116P and V187D (p < 0.001). However, for the A116P/S1238Y and V187D/S1238Y mutants, without the S1238Y mutation, both 1 and 5 μM glibenclamide significantly increased surface expression of the A116P and V187D trafficking mutants (p < 0.001). However, for the A116P/S1238Y and V187D/S1238Y mutants, both 1 and 5 μM glibenclamide did not lead to a statistically significant increase in surface expression, whereas 5 μM glibenclamide did (p < 0.01). For both B and C, each bar is the mean ± S.E. of three to five independent experiments.

FIGURE 5. Impact of the Y230A mutation on the effectiveness of sulfonylureas and glinides to rescue K<sub>ATP</sub> channel trafficking defects in the presence of Kir6.2. The A116P or V187D trafficking mutation was introduced onto the WT- or Y230A-fSUR1 background. Each of these fSUR1 constructs was cotransfected with Kir6.2 into COS<sub>6</sub> cells and cell surface expression of the FLAG epitope was quantified by chemiluminescence assays. Cells were treated with or without 5 μM glibenclamide (A), 10 μM repaglinide (B), or 300 μM tolbutamide (C), for 24 h prior to the assay. All three drugs significantly increased surface expression of the trafficking mutants on the WT background (p < 0.001). On the Y230A mutation background, however, the glibenclamide rescue effect was attenuated although still significant (p < 0.01), whereas the effects of repaglinide and tolbutamide were both abolished. Note that in B, 10 μM repaglinide was shown as this concentration gave the maximal effect, although 200 nM and 1 μM were also tested and gave similar results (not shown). Each bar is the mean ± S.E. of three to five independent experiments.
we observed reduced tolbutamide block at 10 and 300 μM, indicating a role of Tyr230 in mediating tolbutamide response both in the context of channel trafficking rescue and channel activity block. As expected, Y230A also reduced glibenclamide block at 10 nM and 1 μM. However, Y230A did not render glibenclamide inhibition reversible; this is in contrast to the S1238Y mutation that caused reduced block by 10 nM glibenclamide and rendered the block reversible upon washout (Fig. 7A) as reported previously by others (20). Furthermore, consistent with the Ser1238 residue being critical for high affinity tolbutamide binding, we observed little tolbutamide block at 10 μM and reduced block at 300 μM in the S1238Y mutant (the residual block at 300 μM likely is mediated by the low affinity site in Kir6.2) (20, 31). Finally, combining Y230A and S1238Y had a greater effect on preventing tolbutamide and glibenclamide block than either mutation alone (Fig. 7). Together, the above results show that Tyr230 not only participates in conferring the effect of glibenclamide but also is involved in conferring the effect of tolbutamide on channel trafficking rescue and channel activity block.

The Role of Kir6.2 in Sulfonylurea Rescue of Channel Trafficking Defects—The data we presented so far demonstrate that intact sulfonylurea binding sites in SUR1 are necessary for effective rescue of the A116P- or V187D-SUR1 trafficking mutants by sulfonylureas. In addition to high affinity binding to SUR1, sulfonylureas are known to interact with Kir6.2 with low affinity (31, 32). To investigate the role of Kir6.2 in sulfonylurea rescue of the A116P- or V187D-SUR1 trafficking mutants, we took advantage of the fact that inactivation of the -RKR- ER retention/retrieval motif by mutation to AAA (referred to as WTAAA in Fig. 8A) in SUR1 allows SUR1 to traffic to the cell surface without co-expression of Kir6.2 (29). If sulfonylurea binding to SUR1 is sufficient to correct the defect caused by the A116P or V187D mutations, we expect sulfonylureas to improve the surface expression of the A116PAAA and V187DAAA SUR1 constructs, which expressed efficiently at the cell surface both in the presence or absence of Kir6.2. Chemiluminescence assay results show that the A116PAAA and V187DAAA SUR1 exhibited a very low level of cell surface expression whether Kir6.2 was present or absent. These results are consistent with the A116P and V187D mutations causing defects in the SUR1 protein itself. Interestingly, although sulfonylurcnergic drugs were able to markedly increase surface expression of the A116PAAA and V187DAAA SUR1 when Kir6.2 was co-expressed, they failed to do so when Kir6.2 was absent. Patch clamp recordings confirm that WTAAA, A116PAAA, and V187DAAA-SUR1 each form functional channels with Kir6.2 (supplemental Fig. S4), indicating that the surface fSUR1AAA detected in the chemiluminescence assay is indeed coassembled with Kir6.2 as functional channels. The above results led us to conclude that Kir6.2 is required for sulfonylureas to exert their rescue effect on KATP channels with trafficking defects caused by SUR1 mutations.
Recently, we reported that several mutations in Kir6.2 identified from patients with permanent neonatal diabetes also reduce expression of KATP channels in addition to affecting channel ATP sensitivity (10). We further showed that sulfonylureas increased surface expression of some of these mutant channels (10). To test whether the effect of sulfonylureas on surface expression of mutant Kir6.2 channels is in turn dependent on intact sulfonylurea binding in the SUR1 subunit, we examined surface expression of three Kir6.2 mutants, R201H, R201C, or I296L, co-expressed with WT or S1238Y SUR1, in cells treated with or without 300 μM tolbutamide. As shown in Fig. 8B, eliminating the high affinity tolbutamide site in SUR1 by the S1238Y mutation rendered tolbutamide much less effective in rescuing the Kir6.2 mutants, although some residual
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Effect was still observed. Thus, both SUR1 and Kir6.2 are required for sulfonylureas to overcome channel trafficking defects caused by mutations in either subunit.

**DISCUSSION**

The oral hypoglycemic agents sulfonylureas have long been used to treat patients with type II diabetes (4, 11, 12), and more recently neonatal diabetes caused by mutations in the $K_{\text{ATP}}$ channel subunits (33–35). The primary therapeutic action of sulfonylureas is to inhibit $\beta$-cell $K_{\text{ATP}}$ channel activity via high affinity binding to SUR1. Our recent study has shown that in addition to inhibiting channel activity, sulfonylureas also markedly increase surface expression of channels carrying certain CHI-causing SUR1 mutations that would otherwise be retained in the ER (23). As these mutant channels exhibit relatively normal function upon rescue to the surface in patch clamp experiments (23), their physiological function may be restored in patients by exploiting the chaperoning activity of sulfonylureas. Such translational application would require that we understand the mechanism by which sulfonylureas rescue $K_{\text{ATP}}$ channel trafficking defects. The studies presented here begin to address the issue by testing specifically whether sulfonylureas exert the effect on channel trafficking via direct binding to the $K_{\text{ATP}}$ channel complex and analyzing the structural moieties of sulfonylureas that are important for the rescue effect. These are important steps toward understanding the mechanism given that sulfonylureas have been reported to have other cellular targets (36–39) and there are many sulfonylurea-related hypoglycemia agents in use clinically. Our results demonstrate that mutations in SUR1 previously reported to abolish or reduce sulfonylurea binding also abolish or reduce the ability of sulfonylureas to rescue channel trafficking defects caused by the A116P or V187D SUR1 mutations and that both the sulfonylurea and benzoamido moieties of glibenclamide contribute to the rescue effect. The effects of binding mutations on channel response to sulfonylurea block correlate qualitatively with their effects on channel trafficking rescue by sulfonylureas. Interestingly, we found that Kir6.2 co-expression is necessary to see an effect of sulfonylureas on surface expression of the mutant SUR1. Conversely, rescue of mutant Kir6.2 surface expression relies on intact sulfonylurea binding sites in SUR1. Together, our data provides strong evidence that sulfonylureas rescue $K_{\text{ATP}}$ channel trafficking defects by direct interactions with the channel complex.

**Mechanisms by Which Sulfonylureas Correct $K_{\text{ATP}}$ Channel Trafficking Defects**—It is well documented that trafficking defects of some membrane proteins caused by protein misfolding, including CFTR, human P-glycoprotein, HERG channels, and $\beta$-glucosidase can be overcome by treating cells with small molecules that act as pharmacological chaperones to facilitate protein folding (40, 41). A reasonable hypothesis for how sulfonylureas improve surface expression of the A116P and V187D mutants is that A116P or V187D cause SUR1 misfolding and sulfonylureas, upon binding to the mutant SUR1, help it to adopt the correct conformation. Our results that the A116P or V187D mutations are sufficient to prevent trafficking of TMD0 as well as SUR1$_{\text{AAA}}$ to the cell surface support the idea that the A116P or V187D mutations have an effect on the folding/processing of SUR1 protein itself. The fact that intact sulfonylurea binding sites in SUR1 are necessary for the rescue effect on channel trafficking is also consistent with our hypothesis. However, the finding that sulfonylureas fail to improve surface expression of A116P$_{\text{AAA}}$-SUR1 and V187D$_{\text{AAA}}$-SUR1 in the absence of Kir6.2 suggests the mechanism is likely more complicated. Kir6.2 is known to bind with sulfonylureas by itself, albeit with very low affinity (32); and to increase the binding affinity of sulfonylurea and glinide to the $K_{\text{ATP}}$ channel complex (14, 21, 42). It is possible that the presence of Kir6.2 contributes to sulfonylurea binding to allow the drug to correct mutant SUR1 folding. Alternatively, the involvement of the Kir6.2 subunit could be independent of its role in sulfonylurea...
binding but have more to do with an effect on SUR1 folding as the subunits coassemble, as has been reported for voltage-gated potassium channels where tertiary folding and oligomerization of channel subunits are coupled (43). It is also worth pointing out that TMD0 harboring the A116P and V187D mutations have been shown to not coimmunoprecipitate with Kir6.2 (27), suggesting weakened association between mutant SUR1 and Kir6.2. If sulfonylurea rescue mutant channel expression by restoring interactions between mutant SUR1 and Kir6.2, it would explain the requirement of Kir6.2 coexpression. Interestingly, we recently reported that not only do sulfonylureas correct the trafficking defects caused by certain CHI-associated SUR1 mutations, they also improve the surface expression of channels carrying certain PNDM-associated Kir6.2 mutations (10). Again, our results that the effect of sulfonylureas on surface expression of these Kir6.2 mutants is dependent on the sulfonylurea binding site in SUR1 further support the notion that sulfonylureas likely rescue channel trafficking defects by acting on the SUR1–Kir6.2 complex rather than individual subunits.

Implications on Sulfonylurea Binding Sites—Many studies have been carried out to map the sulfonylurea binding sites in $K_{ATP}$ channels, in particular the high affinity binding site(s) in SUR1. Binding of tolbutamide, a first-generation short chain sulfonylurea, is thought to involve transmembrane segments 14–16, which likely represents the “A site” in the classic pharmacophore model (Fig. 1). Binding of glibenclamide, a second generation sulfonylurea that contains an additional aromatic moiety attached to the short chain via a carboxamidohio group, appears to involve not only the A site but also a “B site” that likely involves the cytoplasmic loop between TMD0 and TMD1 (21, 44). Consistent with this picture, we found that mutation S1238Y in the proposed A site abolished the effect of tolbutamide and attenuated the effect of glibenclamide on the trafficking mutants, whereas mutation Y230A in the proposed B site rendered repaglinide ineffective and glibenclamide less effective in rescuing the trafficking mutants. To our surprise, mutation Y230A in the proposed B site also prevented tolbutamide from rescuing the trafficking mutants; whereas this may suggest a role of Tyr$^{230}$ in tolbutamide binding, an alternative possibility is that the residue is involved in post-binding events that are important for the rescue effect of the drug. In this regard, the cytoplasmic loop where Tyr$^{230}$ resides has been proposed to interact with the N terminus of Kir6.2 to control channel activity; deletion of the N terminus of Kir6.2 causes uncoupling between tolbutamide binding in SUR1 and channel activity block (19, 28, 45–47). The Y230A mutation might abolish the tolbutamide rescue effect on the trafficking mutants by disrupting the cross-talk between SUR1 and Kir6.2, which we have shown to be necessary for the sulfonylurea rescue effects. Interestingly, we observed parallel effects of S1238Y and/or Y230A on the ability of sulfonylureas to rescue trafficking mutants and block channel activity, suggesting the two processes likely share similar transduction mechanisms. Of note, the effective concentrations for channel trafficking rescue are in general higher than for channel activity block, perhaps because in the former case the drug has to reach deep into the cell to access the ER-retained mutant channels.

In summary, sulfonylureas as well as glinides increase surface expression of ER-retained mutant $K_{ATP}$ channels containing CHI-associated SUR1 or PNDM-associated Kir6.2 mutations by interacting with the mutant channel complex. The process of $K_{ATP}$ channel subunit biosynthesis, assembly, and trafficking is still poorly understood (48–50). Whether channel assembly occurs cotranslationally or post-translationally, at which step sulfonylureas interact with the channel proteins, and what conformational changes in the channel proteins occur upon drug binding are important questions to address in the future. Although we used the A116P and V187D-SUR1 mutations as two examples for probing the mechanism by which sulfonylureas rescue channel trafficking defect, we have found many more CHI-causing $K_{ATP}$ mutants with trafficking defects to respond to sulfonylurea rescue.3 Our findings are therefore applicable to a growing number of naturally occurring channel mutations whose trafficking defects could be targeted for therapy.

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