Destabilization of ATP-sensitive Potassium Channel Activity by Novel KCNJ11 Mutations Identified in Congenital Hyperinsulinism*

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The inwardly rectifying potassium channel Kir6.2 is the pore-forming subunit of the ATP-sensitive potassium (K
\(_{\text{ATP}}\)) channel, which controls insulin secretion by coupling glucose metabolism to membrane potential in \(\beta\)-cells. Loss of channel function because of mutations in Kir6.2 or its associated regulatory subunit, sulfonylurea receptor 1, causes congenital hyperinsulinism (CHI), a neonatal disease characterized by persistent insulin secretion despite severe hypoglycemia. Here, we report a novel \(K_{\text{ATP}}\) channel gating defect caused by CHI-associated Kir6.2 mutations at arginine 301 (to cysteine, glycine, histidine, or proline). These mutations in addition to reducing channel expression at the cell surface also cause rapid, spontaneous current decay, a gating defect we refer to as inactivation. Based on the crystal structures of Kir3.1 and KirBac1.1, Arg-301 interacts with several residues in the neighboring Kir6.2 subunit. Mutation of a subset of these residues also induces channel inactivation, suggesting that the disease mutations may cause inactivation by disrupting subunit-subunit interactions. To evaluate the effect of channel inactivation on \(\beta\)-cell function, we expressed an alternative inactivation mutant R301A, which has equivalent surface expression efficiency as wild type channels, in the insulin-secreting cell line INS-1. Mutant expression resulted in more rapid, spontaneous current decay, a gating defect we refer to as inactivation. In vitro studies have shown that these molecules stimulate channel activity and reduce channel sensitivity to ATP inhibition by interacting with Kir6.2 (5–8). Although it remains to be determined if membrane phosphoinositides or long-chain acyl-CoAs are active physiological regulators of \(K_{\text{ATP}}\) channel activity (9), evidence indicates that these lipid molecules are necessary for sustaining channel activity in vivo (10, 11).

Inwardly rectifying potassium (Kir) channels are important for governing the resting membrane potential in a wide variety of cell types (1). In the islet \(\beta\)-cell, Kir6.2 complexes with the sulfonylurea receptor 1 (SUR1) to form the ATP-sensitive potassium (\(K_{\text{ATP}}\)) channel which regulates membrane potential according to the energetic state of the cell, thereby mediating glucose-stimulated insulin secretion (2–4). The gating properties that are critical for the physiological function of \(K_{\text{ATP}}\) channels are their sensitivity to intracellular nucleotides ATP and ADP, whose concentrations fluctuate as glucose levels vary. Both Kir6.2 and SUR1 subunits participate in nucleotide regulation of the channel; ATP inhibits channel activity by binding to the Kir6.2 subunit, whereas Mg\(^{2+}\)-complexed ATP and ADP stimulate channel activity by interacting with SUR1. As glucose concentrations rise, \(K_{\text{ATP}}\) channels are driven to closure by the increase in ATP and decrease in ADP levels, resulting in membrane depolarization, activation of voltage-gated calcium channels, and insulin secretion. On the other hand, a fall in glucose concentrations promotes \(K_{\text{ATP}}\) channel opening to stop insulin secretion. Other molecules that have emerged from recent studies as important players in channel gating are membrane phosphoinositides and long-chain acyl-CoAs. In vitro studies have shown that these molecules stimulate channel activity and reduce channel sensitivity to ATP inhibition by interacting with Kir6.2 (5–8). Although it remains to be determined if membrane phosphoinositides or long-chain acyl-CoAs are active physiological regulators of \(K_{\text{ATP}}\) channel activity (9), evidence indicates that these lipid molecules are necessary for sustaining channel activity in vivo (10, 11).

Mutations in the Kir6.2 gene KCNJ11 and the SUR1 gene ABCC8 that abrogate the function of \(K_{\text{ATP}}\) channels either by preventing expression of functional channels at the cell surface or by faulty gating regulation results in the insulin secretion disorder congenital hyperinsulinism characterized by persistent \(\beta\)-cell depolarization and insulin secretion despite severe hypoglycemia (12–14). In contrast, mutations leading to an overall enhancement of channel activity by reducing channel sensitivity to ATP inhibition, increasing channel stimulation by Mg\(^{2+}\) nucleotides, or increasing surface channel numbers...
blunt glucose-stimulated insulin secretion and cause neonatal diabetes (15–17). In congenital hyperinsulinism, the most common channel gating defect is diminished response to nucleotide stimulation, which has so far only been associated with SUR1 mutations (18, 19). Recently, we reported another gating defect underlying CHI; that is, reduced channel response to membrane phosphoinositides and long-chain acyl-CoAs in channels bearing a Kir6.2 missense mutation F55L (11). Characterization of channel defects caused by disease mutations has advanced our understanding of the structure-functional relationship of the channel and the gating properties that are important for the physiological function of the channel. In this study we investigated the mechanisms by which four CHI-associated mutations on residue Arg-301 of Kir6.2, R301C, R301G, R301H, and R301P, render channel dysfunction. We found that these mutations not only reduce surface expression efficiency of the channel but also cause a gating defect characterized by rapid spontaneous decay of channel activity in the absence of ATP and recovery of channel activity upon subsequent exposure and removal of ATP. In an earlier study we have reported a similar gating defect, referred to as inactivation, induced by disruption of an ion pair formed by two oppositely charged residues from neighboring Kir6.2 subunits in the Kir6.2 tetramer (20). We hypothesized that mutations at the Arg-301 residue may also cause channel inactivation by disrupting interactions between adjacent Kir6.2 subunits based on the predicted location of the Arg-301 residue in the KirBac1.1 and Kir3.1 cytoplasmic domain structures. Mutagenesis studies yielded results that are consistent with the idea that Arg-301 may interact with multiple residues in the adjacent Kir6.2 subunit to stabilize channel activity. Moreover, we demonstrate that the channel inactivation defect itself is sufficient to recapitulate abnormal β-cell membrane electrical properties and insulin secretion expected of CHI. The results identify a novel \( K_{\text{ATP}} \) channel gating defect contributing to congenital hyperinsulinism and support the importance of Kir6.2 intersubunit interactions in channel gating and function.

**MATERIALS AND METHODS**

**Clinical and Genetic Analyses**—Tests of acute insulin responses to calcium (2 mg/kg), leucine (15 mg/kg), glucose (0.5 gm/kg), and tolbutamide (25 mg/kg) sequentially infused intravenously at intervals of 1 h were carried out as previously described (21). Peripheral blood was obtained for isolation of genomic DNA from the probands and family members. Direct sequencing of DNA from the patient and family members was done as previously described (21). Written informed consent was obtained from all patients and family members of this study.

**Molecular Biology**—Rat Kir6.2 cDNA is in pCDNAI/Amp vector and SUR1 in pECE. Site-directed mutagenesis was carried out using the QuikChange site-directed mutagenesis kit (Stratagene), and the mutation was confirmed by sequencing. Mutant clones from at least two independent PCR reactions were analyzed to avoid false results caused by undesired mutations introduced by PCR. Construction of adenovirus carrying R301A, R301H, or R301P Kir6.2 cDNA was as described previously (10). The fSUR1 recombinant adenovirus was constructed using a modified pShuttle plasmid (AdEasy kit, Stratagene) containing a tetracycline-inducible promoter (22). Recombinant viruses were amplified in HEK293 cells and purified according to the manufacturer’s instructions (10).

**Virus Infection**—INS-1 cells clone 832/13 (kindly provided by Dr. Christopher Newgard) (23) were plated in 24-well plates and cultured for 24 h in RPMI 1640 with 11.1 mM D-glucose (Invitrogen) supplemented with 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. For Kir6.2 expression, recombinant adenoviruses containing wild type (WT) or Arg-301 mutant Kir6.2 with desired titers were used to infect cells as described previously (10). For fSUR1 expression, cells were co-infected with equal amounts of two recombinant adenoviruses, one encoding a tetracycline-inhibited transactivator (tTA) and the other a tTA-regulated gene expressing fSUR1 (24). Cells at ~50% confluent density were washed once with phosphate-buffered saline and then incubated for 3 h at 37 °C in RPMI supplemented with 10% fetal bovine serum and a mixture of viruses with the multiplicity of infection (m.o.i.) of each virus determined empirically to obtain the desired protein expression level. Medium was then replaced with fresh growth medium, and the cells were incubated at 37 °C until reaching appropriate density for the various experiments.

**Western Blotting and Chemiluminescence Assay**—Cell surface expression level of the mutant channel was assessed by Western blot and by quantitative chemiluminescence assays using a SUR1 that was tagged with a FLAG epitope (DYKD-DDDK) at the N terminus (fSUR1), as described previously (25). COSm6 cells grown in 35-mm dishes were transfected with 0.4 μg of rat Kir6.2 and 0.6 μg of fSUR1 and lysed 48–72 h post-transfection in 20 mM HEPES, pH 7.0, 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40 (IGAPEL) with Complete TR protease inhibitors (Roche Applied Science). Proteins in the cell lysate were separated by SDS/PAGE (7.5% for SUR1 and 12% for Kir6.2), transferred to nitrocellulose membrane, analyzed by incubation with appropriate primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences), and visualized by enhanced chemiluminescence (Super Signal West Femto; Pierce). The primary antibodies used were M2 mouse monoclonal anti-FLAG antibody for fSUR1 (Sigma) and rabbit polyclonal anti-Kir6.2 for Kir6.2 (Santa Cruz Biotechnology, Santa Cruz, CA). For chemiluminescence assays cells were fixed with 2% paraformaldehyde for 30 min at 4 °C. Fixed cells were preblocked in phosphate-buffered saline + 0.1% BSA for 30 min, incubated in M2 anti-FLAG antibody (10 μg/ml) for 1 h, washed 4 × 30 min in phosphate-buffered saline + 0.1% BSA, incubated in horseradish peroxidase-conjugated anti-mouse (Jackson Immuno-Research Laboratories, Inc., 1:1000 dilution) for 20 min, and washed again 4 × 30 min in phosphate-buffered saline + 0.1% BSA. Chemiluminescence of each dish was quantified in a TD-20/20 luminometer (Turner Designs) after a 15-s incubation in Power Signal Elisa lum囷lor solution (Pierce). All steps after fixation were carried out at room temperature.

**Electrophysiology**—Patch clamp recordings were performed in the inside-out or whole-cell configurations as previously
described (25). For inside-out recordings in COSm6 cells, cells were transfected with cDNA encoding WT or mutant channel proteins as well as cDNA for the green fluorescent protein to help identify transfected cells. Patch clamp recordings were made 36–72 h post-transfection. Micropipettes were pulled from non-heparinized Kimble glass (Fisher Scientific) with resistance typically ~1–2 megaohms. The bath (intracellular) and pipette (extracellular) solution (K-INT) had the following composition: 140 mM KCl, 10 mM K-HEPES, 1 mM K-EGTA, pH 7.3. To minimize channel rundown, 1 mM EDTA was included in K-INT for most experiments (referred to as K-INT/EDTA) (20). ATP was added as the potassium salt. All currents were measured at a membrane potential of −50 mV (pipette voltage = +50 mV) and inward currents shown as upward deflections.

For experiments using INS-1 cells, cells were transduced using the various recombinant viruses as described above. Inside-out patch clamp recording was carried out as in COSm6 cells. Whole-cell patch clamp recording was used to measure INS-1 cell membrane potential at different glucose concentrations (10). One day after virus infection, INS-1 cells were pre-incubated in 5 mM glucose RPMI medium for 18 h followed by incubation in 3 or 12 mM glucose for 3 h before recording. During recording, cells were bathed in Tyrode solution consisting of 137 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 0.5 mM MgCl2, 5 mM HEPES, 3 mM NaHCO3, and 0.16 mM NaH2PO4 with 3 or 12 mM glucose. The pipette solution contained 10 mM KCl, 130 mM potassium gluconate, 10 mM HEPES, 1 mM EGTA, 1 mM MgCl2, and 1 mM ATP for 3 mM glucose bath solution or 3 mM MgCl2 and 5 ATP for 12 mM glucose bath solution.

86Rb+ Efflux Assay—COSm6 or INS-1 cells were plated in 6-well tissue culture plates and transfected with SUR1 and Kir6.2 cDNA or infected with viruses as described above. Cells were incubated for 12 h in culture medium containing 86RbCl (1 μCi/ml) 24 h post-transfection or infection. Before measurement of 86Rb+ efflux, cells were incubated for 5 min at room temperature in Krebs-Ringer solution with metabolic inhibitors (2.5 μg/ml oligomycin and 1 mM 2-deoxy-D-glucose). At the select time points the solution was aspirated from the cells and replaced with fresh solution. At the end of a 40-min period, cells were lysed. The 86Rb+ in the aspirated solution and the cell lysate was counted. The percentage efflux at each time point was calculated as the cumulative counts in the aspirated solution divided by the total counts from the solutions and the cell lysates.

Insulin Secretion Assay—INS-1 cells were seeded in 24-well tissue culture plates at ~5 × 10⁵/well, cultured for ~24 h, and 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. Insulin secretion was assayed in HEPES balanced salt solution consisting of 114 mM NaCl, 4.7 mM KCl, 1 mM MgCl2, 1.2 mM KH2PO4, 1.16 mM MgSO4, 20 mM HEPES, 2.5 mM CaCl2, 25.5 mM NaHCO3, and 0.2% bovine serum albumin (pH 7.2). Cells were washed twice with prewarmed (37 °C) HEPES balanced salt solution (HBSS) buffer with 3 mM glucose followed by 2-h incubation in the same buffer before stimulation with 0.8 ml/well pre-warmed HBSS 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 and insulin secretion capacity. The resulting value was then normalized to that observed in uninfected cells at 3 mM glucose and expressed as -fold increase in insulin secretion.

Data Analysis—Data were analyzed using pCLAMP software (Axon Instrument). Off-line analysis was performed using Microsoft Excel programs. Statistical analysis was performed using independent two-population two-tailed Student’s t test.

RESULTS

Identification of Four Missense Mutations at Residue Arg-301 of Kir6.2 and Clinical Summary on the Patients—The four Kir6.2 Arg-301 mutations were found in patients with hyperinsulinism who presented in the newborn period with severe hypoglycemia; all four children failed to respond to medical therapy with the KATP channel agonist, diazoxide (Table 1). Two of the patients (R301H and R301C) had focal lesions due to isodisomy for a paternally inherited mutation in a clone of pancreatic cells. The R301H mutation has been previously described (26). A third patient (R301G) is presumed to have a focal lesion since she had a single, paternally derived mutation; however, surgery was not performed since she was adequately managed using a long-acting somatostatin analog, octreotide. The fourth patient had diffuse disease due to a homozygous R301P mutation and required pancreatectomy to manage his hypoglycemia.

Effects of Arg-301 Mutations on Surface Expression of KATP Channels in COS Cells—To evaluate the molecular consequence of these mutations, we first examined whether the mutation affects channel processing and expression when reconstituted in COSm6 cells. For this, cells were cotransfected with WT or mutant Kir6.2 cDNA and the cDNA encoding a SUR1 that had been tagged at the extracellular N terminus with a FLAG epitope (herein referred to as fSUR1), which permits detection of fully assembled channel complexes expressed at the plasma membrane. Western blots showed that the steady-state mutant Kir6.2 protein level for R301H and R301P is reduced compared with WT Kir6.2 (Fig. 1A). Moreover, fSUR1 co-expressed with the R301G, R301H, or R301P showed significantly reduced maturation efficiency as judged by the level of the complex-glycosylated band (Fig. 1A), which represents the fraction of the protein that has exited the endoplasmic reticulum and moved passed the medial Golgi where modification of N-linked glycosylation occurs. The results indicate that these mutant channels likely have biogenesis and surface expression defects. On the other hand, the R301C mutation appears to have little effect.

### TABLE 1

| Type of patient | Mutation | Allele | Surgery | Diazoxide response |
|----------------|----------|--------|---------|-------------------|
| Focal          | R301H    | Paternal | Yes     | No                |
| Diffuse        | R301P    | Homozygous | Yes     | No                |
| Focal          | R301C    | Paternal | Yes     | No                |
| Presumed focal | R301G    | Paternal | No      | No                |
on the steady-state level of the Kir6.2 or the processing efficiency of SUR1. To better quantify channel expression at the plasma membrane, we monitored the level of channel present at the cell surface using chemiluminescence assays described under "Materials and Methods." Results from these experiments showed that the R301G, R301H, and R301P were expressed poorly at 26.3 ± 5.9, 19.6 ± 4.0, and 4.4 ± 2.6% (n = 3) that of the level of WT channels, respectively (Fig. 1B). Surface expression of R301C mutation, on the other hand, was only mildly reduced compared with WT (at 76.5 ± 4.3%, n = 3). The reduction in surface expression observed in these mutants is expected to reduce channel function and contributes to the hyperinsulinism disease phenotype.

*Missense Mutations at Arg-301 Destabilize K<sub>ATP</sub> Channel Activity—*To further assess channel expression levels using functional assays and to test whether the mutation also affects channel gating, we performed inside-out patch clamp recording of mutant channels expressed in COSm6 cells. In accordance with surface expression data obtained by chemiluminescence assays, the R301G, R301H, and R301P mutants had very small currents, and the R301C mutant activity was reduced. Interestingly, in patches where currents were detected, the mutants all exhibited spontaneous, rapid current decay upon membrane excision into K-INT/EDTA (Fig. 2, A–E, compared with WT in Fig. 2A). We have previously described a similar gating behavior, referred to as inactivation, in a number of recombinant Kir6.2 mutants, including R192A, E227A, E229A, R301H, and R314A (27). Inactivation is distinct from channel rundown frequently observed in WT K<sub>ATP</sub> channels. We have shown in a prior study that the two phenomena can be distinguished by including 1 mM EDTA in the K-INT bath solution; whereas EDTA dramatically minimizes rundown in WT channels, it has no effect on inactivation seen in the mutants (20). The single channel conductance for all mutant channels remains the same as WT channels (~70 picoSiemens at −50 mV membrane potential with symmetrical K-INT solution; recordings not shown) (11). The average peak current size immediately after patch excision for the various Arg-301 mutants normalized to WT channel activity are as follows: 38.3 ± 7.4% for R301C, 2.9 ± 0.9% for R301G, 3.7 ± 0.4% for R301H, and 4.7 ± 1.8% for R301P; n = 7, 16, 13, and 15, respectively. These values are in general lower than expected based on surface expression data possibly because some of the channels were already in the inactivated state at the time of patch excision.

As the inactivation mutants we described before (27), the Arg-301 disease mutants could recover from inactivation by exposing the patch to inhibitory ATP (5 mM) and subsequent removal of ATP recovered the channels from inactivation (not done for the R301G mutation). Figure 2 shows the extent of rundown for various mutants compared to WT channels. As the inactivation mutants we described before (27), the Arg-301 disease mutants could recover from inactivation by exposing the patch to inhibitory ATP (5 mM) and subsequent removal of ATP recovered the channels from inactivation (not done for the R301G mutation). Figure 2 shows the extent of rundown for various mutants compared to WT channels. In K-INT/EDTA solution, WT currents were stable for the duration of the recording. In contrast, the currents from mutant channels decayed rapidly after patch excision. 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**KCNJ11 Mutation, K$_{ATP}$ Channel Inactivation, and Hyperinsulinism**

Mutational Analysis of Residues That Potentially Form Intersubunit Interactions with Arg-301 to Stabilize Channel Activity—

One striking feature shared by mutations that cause K$_{ATP}$ channel inactivation is that the mutated residues are all located at the Kir6.2 subunit-subunit interface (20, 27), based on the crystal structure of two inwardly rectifying channels, Kir3.1 and KirBac1.1, which are highly homologous to Kir6.2 (28, 29). Indeed, we have previously demonstrated that Glu-229 and Arg-314 form an intersubunit ion pair that is critical for maintaining the stability of channel activity (20). Mutations that disrupt the ionic interaction cause channel inactivation, whereas charge swap mutations at the two residues or cross-linking of cysteine residues engineered into the two positions restore channel stability. This led us to hypothesize that the disease mutations at Arg-301 as well as the previously published recombinant mutation R301A (27) may cause channel inactivation by disrupting Kir6.2 intersubunit interactions. To test this idea, we performed mutational analysis of residues that are predicted to interact with Arg-301 in the neighboring Kir6.2 subunit according to the Kir3.1 cytoplasmic domain structure or the KirBac1.1 structure (see Fig. 3 and Table 2). The most prominent candidate to form intersubunit interaction with Arg-301 is Glu-292, which in both the Kir3.1 and KirBac1.1 structures lies closely to Arg-301 of the neighboring subunit (within 7 and 4 Å, respectively) (28, 29) (Fig. 3). We, therefore, mutated Glu-292 to alanine, glutamine, or cysteine and analyzed mutant channels expressed in COS cells by inside-out patch clamp recording. Although the E292C mutant did exhibit inactivation, the E292A and E292Q mutants surprisingly were indistinguishable from WT channels (Fig. 4), suggesting that disruption of charge interaction is insufficient to cause inactivation. Because the E292C channel did show inactivation, we sought to determine whether it might be possible to cross-link cysteine residues engineered into both Glu-292 and Arg-301 positions to restore channel stability, a strategy that we have successfully employed before to demonstrate the physical interactions between Glu-229 and Arg-314 (20). However, when cysteine was introduced into both 292 and 301 positions, no channel activity could be detected. Western blot and chemiluminescence experiments confirmed that the mutant was not expressed at the cell surface (not shown). The lack of expression precluded the possibility of the cysteine cross-linking experiment. Another double mutation E292R/R314E also failed to express at the cell surface. The above data, although consistent with a role of Glu-292 in maintaining channel stability, do not provide information about the nature of the interaction between Glu-292 and Arg-301 that is important for channel activity.

Besides Glu-292, several other residues in the vicinity (limited to within 7 Å for this study) of Arg-301 could potentially mediate subunit-subunit interactions. According to the Kir3.1 structure, these include Ile-211, Ser-212, and Phe-250, and according to the KirBac1.1 structure, they include Ser-208, Met-209, Ile-210, and Leu-251, Val-252, Ala-253, and Val-290 (some are shown in Fig. 3). We examined select residues from this potential list and found that mutation of Ser-208, Phe-250, and Leu-251 to alanine or cysteine gave rise to channels that inactivated, whereas mutation of Ser-212 and Val-252 had no effect, and mutation of Ile-210 did not give rise to detectable channel activity; these results are summarized in Table 2, and current traces shown in Fig. 5. Collectively, the mutagenesis studies indicate that many residues situated at Kir6.2 subunit-subunit interface near the Arg-301 residue are involved in stabilizing channel activity and are in line with our hypothesis that the Arg-301 disease mutations cause the inactivation gating defect likely by disrupting a web of intersubunit chemical interactions that are necessary for normal channel gating.

Consequence of K$_{ATP}$ Channel Inactivation in β-Cell Function—An important question arising from the observation that the Arg-301 mutations cause K$_{ATP}$ channel inactivation is whether the inactivation gating defect contributes to β-cell dysfunction and the CHI disease phenotype. However, the reduced surface expression efficiency of the Arg-301 disease mutants based on results obtained in COSm6 cells would make it diffi-
cult to assess the effect of channel inactivation gating defect alone on β-cell function. To circumvent this problem, we included in our analysis the R301A mutant, which exhibits inactivation like the Arg-301 disease mutants (27) but is well expressed at the cell surface in COS cells as reflected in Western blot and patch clamp recording experiments (see Fig. 7A). Quantification of surface channel expression by chemiluminescence assays showed that the R301A mutant is expressed at 95.1 ± 6.7% that of the WT level (n = 5).

To determine whether channel inactivation affects β-cell membrane properties, we expressed the mutants in the rat insulinoma cell line INS-1. Because INS-1 cells are difficult to transfect using conventional lipid-based DNA delivery protocols, we used the recombinant adenovirus transduction method for expression. To ensure there is sufficient SUR1 protein to form a channel complex with the exogenously expressed Kir6.2, INS-1 cells were co-transduced with fSUR1 and WT or mutant Kir6.2 recombinant viruses (24). Cells transduced with fSUR1 and WT or mutant Kir6.2 viruses exhibited significantly increased channel expression at the cell membrane compared with uninfected INS-1 cells, based on current density in inside-out patch clamp recordings (137 ± 29 pA in uninfected cells versus 1407 ± 301 pA in cells infected with WT fSUR1 and Kir6.2, n = 15–19). Consistently, a Western blot of fSUR1 showed an abundant complex-glycosylated form of the protein (Fig. 6A). In contrast, cells transduced with an equivalent titer of R301H or R301P mutant KATP viruses exhibited similar current density as uninfected cells, and little channel inactivation was observed (not shown). When the m.o.i. of the mutant Kir6.2 was increased by 10–20-fold, some expression of the R301H mutant was detected, as evident from the appearance of the upper fSUR1 band in Western blot and current inactivation in excised patches; however, expression of the R301P mutant at the cell surface remained undetectable (Fig. 6). These results are consistent with our finding in COSm6 cells shown in Fig. 1. Of note, at these high m.o.i., the cells appeared unhealthy and were difficult to patch.

We, therefore, proceeded to express the R301A mutant channels in INS-1 cells using viral titers that gave similar protein expression level as WT by Western blots.
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FIGURE 6. Expression of the R301H and R301P mutants in INS-1 cells.
A, Western blots of Kir6.2 and fSUR1 in INS-1 cells transduced with WT fSUR1 and WT, R301H, or R301P Kir6.2 recombinant viruses (see "Materials and Methods"). Note the m.o.i. used for the mutant Kir6.2 was 20-fold higher than WT in order to see a significant signal. Even at this high m.o.i., the amount of complex glycosylated fSUR1 was still barely visible in the R301P mutant. B, representative inside-out patch clamp recordings from uninfected cells and cells infected with the fSUR1/R301H or fSUR1/R301P combination of viruses (at m.o.i. shown in A). The R301H mutant was detected in some cells as evident by channel inactivation, although the R301P mutant remained absent at the surface in most cells as the currents detected were indistinguishable from uninfected cells. Also note at the high m.o.i. used to express the mutants, cells tended to detach from the bottom of the dish, making recordings difficult.

To address how channel inactivation affects \(\beta\)-cell membrane properties, we measured the membrane potential of cells bathed in Tyrode solution containing 3 or 12 mM glucose with pipette solution containing 1 or 5 mM MgATP, respectively, using whole-cell patch clamp recording. Prior studies from our laboratory have shown that the initial break-in potential in whole-cell recording is very similar to that obtained using perforated patching technique (10). The initial break-in potential was, thus, taken as the membrane potential and used for statistical analysis. Fig. 8A shows representative recordings from uninfected cells or cells expressing exogenous WT or R301A channels. To ensure the quality of the recording and the health of the cell, cells were exposed to bath solution containing 250 \(\mu\)M tolbutamide followed by 250 \(\mu\)M diazoxide, which close and open \(K_{\text{ATP}}\) channels, respectively. These experiments show that the membrane potential of uninfected cells and cells expressing exogenous WT channels was significantly more depolarized when cells were exposed to 12 mM glucose than when they were exposed to 3 mM glucose (Fig. 8). At 12 mM glucose, cells frequently fired action potentials as expected. By contrast, cells expressing the R301A mutant exhibited membrane potential that was already significantly more depolarized than the uninfected or WT channel-expressing cells at 3 mM glucose (Fig. 8B). At 12 mM glucose, the difference between the R301A mutant channel-expressing cells and the uninfected or WT channel-expressing control cells was diminished to statistically insignificant level. In fact, the membrane potential appears to be slightly more hyperpolarized than the other two control groups. The more depolarized membrane potential in 3 mM glucose is consistent with the \(\beta\)-cell phenotype expected in congenital hyperinsulinism.

Last, we compared insulin secretion in uninfected INS-1 cells and cells overexpressing equivalent levels of WT or R301A mutant channels at basal (3 mM) or high (12 mM) glucose. As shown in Fig. 9, the R301A mutant-expressing cells secrete more insulin at 3 mM glucose compared with cells expressing equivalent number of WT channels. At 12 mM glucose, however, no statistically significant difference in insulin secretion was observed among the three groups of cells. The above results led us to conclude that inactivation is a \(K_{\text{ATP}}\) channel gating defect that contributes to \(\beta\)-cell dysfunction seen in the disease.

DISCUSSION

The \(K_{\text{ATP}}\) channel gate is guarded by four Kir6.2 subunits. Our previous study has shown that an intersubunit ion pair formed by Glu-229 and Arg-314 at the C-terminal domain of Kir6.2 is essential for maintaining the stability of \(K_{\text{ATP}}\) channel activity (20). Disruption of this ionic interaction leads to \(K_{\text{ATP}}\) channel inactivation characterized by rapid, spontaneous decay of channel activity which can be recovered by exposure to ATP and subsequent removal of ATP (20). In this study we report that four missense mutations on residue Arg-301 of Kir6.2 identified in congenital hyperinsulinism patients cause a similar inactivation gating defect. We show that mutation of several residues that are predicted to form intersubunit interactions...
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The Role of the Arg-301 Residue of Kir6.2 in $K_{ATP}$ Channel Gating—Recently, using homology modeling and ligand docking, Haider et al. (33) proposed Arg-301 as a PIP$_2$ binding residue such as Arg-54, Arg-176, and Arg-177, mutations of which only reduce channel open probability without affecting the stability of channel activity (8, 27, 34). Second, we have previously reported several other mutations that cause channel inactivation but that do not involve residues reported to bind PIP$_2$, including R192A, E229R, and R314A (20, 27). Therefore, other or additional mechanisms must be considered.

Based on our previous work that an intersubunit ion pair formed by Glu-229 and Arg-314 is essential for maintaining channel stability (20), we examined the possibility that Arg-301 mutations may also cause inactivation by disrupting subunit-subunit interactions. Crystal structures of the Kir3.1 cytoplasmic domain and the KirBac1.1 protein show extensive chemical interactions at the subunit-subunit interface. Based on homology mapping to the Kir3.1 and KirBac1.1 structure, Arg-301 of Kir6.2 interacts with several residues in an adjacent Kir6.2. Among the potential interacting residues, Glu-292 is positioned to form an ion pair with Arg-301. We mutated Glu-292 to several different amino acids to test whether the electrostatic interaction between Arg-301 and Glu-292 is the main determinant of $K_{ATP}$ channel stability. Our finding that the E292A and E292Q mutants exhibit WT-like channel stability indicates that the ionic interaction with Arg-301 predicted by the structure is not essential for channel stability. However, the inactivation phenotype observed in the E292C mutant suggests that the Glu-292 residue is indeed involved in stabilizing channel activity. A further mutagenesis study of other potential interacting residues found that Phe-250, Leu-251, Ser-208, and Ser-303 also contribute to channel stability (20).

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arises from our model is whether Arg-301 mutations which cause inactivation also reduce physical association between Kir6.2 subunits. It is interesting to note that although the Arg-301 disease mutations have variegated adverse effects on channel biogenesis, the R301A mutation results in inactivation without affecting channel biogenesis, a process that involves physical co-assembly of the Kir6.2 and SUR1 subunits. This would suggest that disruption of a given subunit-subunit interaction that results in altered channel function might not necessarily manifest as reduced physical association between Kir6.2 subunits in biochemical binding studies. Such a scenario would not be surprising as there are likely multiple interactions at the subunit interface that mediate physical association of subunits, including the Glu-292–Arg-314 ion pair interaction we reported previously (20). Future biochemical studies examining mutants in which one or multiple interactions are interrupted may shed light on the role of each interaction in physical association of Kir6.2 subunits.

How does disruption of Kir6.2 subunit-subunit interactions cause inactivation? We have previously proposed that subunit-subunit interactions may be important to hold the channel in a conformational state that allows the channel to interact with membrane phosphoinositides, which stabilize the channel in the open state. Supporting this idea, inactivation caused by mutation of residues at the subunit-subunit interface can be reversed by application of exogenous PIP2 to the bath solution in inside-out patch clamp recording experiments (20). It is also interesting to note that a common feature of inactivation or loss of open channel stability induced by mutation of residues at the subunit-subunit interface is that inactivation can be recovered by exposing the cytoplasmic domain of the channel to ATP and subsequent removal of ATP. Because the ATP binding pocket is thought to be formed by two adjacent Kir6.2 subunits at the subunit interface, ATP binding could help reestablish the interaction between two neighboring subunits, allowing channels to recover from the inactivated state and open when ATP is subsequently removed. The importance of subunit-subunit interactions in gating likely applies to all Kir channels. In fact, subunit-subunit interactions at homologous amino acid positions in Kir4.1/5.1 and Kir1 channels have been reported to play a role in gating regulation of these channels by pH (35–37).

\[ K_{ATP} \] Channel Inactivation as a Novel Gating Defect Contributing to Congenital Hyperinsulinism—Although \[ K_{ATP} \] channel inactivation has been described in a number of recombinant Kir6.2 mutants (20, 27), the impact of this gating abnormality on the physiological function of the channel remained undetermined. Identification of the four Arg-301 mutations that cause channel inactivation in congenital hyperinsulinism patients suggests that inactivation may contribute to loss of channel function. However, because three of the four mutations (R301G, R301H, or R301P) also severely reduce, and one of them (R301C) slightly reduces surface expression of the channel, we needed to separate the expression defect from the gating defect when determining
the impact of channel inactivation on β-cell function. By examining the effect of another Kir6.2 mutation at the same residue, R301A, which induces channel inactivation without affecting channel surface expression, we were able to establish the functional impact of channel inactivation in insulin-secreting cells. Although for the R301G, R301H, and R301P, channel expression defect is likely the predominant factor underlying loss of channel function, for the R301C mutation channel inactivation likely plays a significant role in causing β-cell defect. Curiously, Girard et al. (38) recently reported identification of a Kir6.2 mutation E229K in transient neonatal diabetes. Glu-229 forms an intersubunit ion pair with Arg-314, and disruption of this ion pair with the E229R mutation induces rapid current decay or inactivation (20). Although E229K also exhibited faster current decay compared with WT channels, the rate is significantly slower than we reported for the E229R mutation (20, 38). Because E229K is a heterozygous mutation in the transient neonatal diabetes TNDM patient, the extent of inactivation is likely further weakened by the WT Kir6.2 present in the channel complex. Additionally, the E229K mutation caused a significant reduction in channel ATP sensitivity. These combined factors likely explain why the Arg-301 mutations cause loss of channel function and CHI, whereas the E229K mutation is associated with transient neonatal diabetes. These studies highlight the complex effects channel mutations may have on channel gating and expression, which in combination determine the disease phenotype manifested in patients.

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