**Analysis of Two KCNJ11 Neonatal Diabetes Mutations, V59G and V59A, and the Analogous KCNJ8 I60G Substitution**

Differences Between the Channel Subtypes Formed with SUR1

Marcus Winkler‡, Rebekka Lutz‡, Ulrich Russ‡, Ulrich Quast‡, and Joseph Bryan§†

From the ‡Department of Pharmacology and Toxicology, Medical Faculty, University of Tübingen, Wilhelmstrasse 56, Tübingen D-72074, Germany and the §Pacific Northwest Diabetes Research Institute, Seattle, Washington 98122

**β-Cell-type K<sub>ATP</sub> channels are octamers assembled from Kir6.2/KCNJ11 and SUR1/ABCC8. Adenine nucleotides play a major role in their regulation. Nucleotide binding to Kir6.2 inhibits channel activity, whereas ATP binding/hydrolysis on sulfonylurea receptor 1 (SUR1) opposes inhibition. Segments of the Kir6.2 N terminus are important for open-to-closed transitions, form part of the Kir ATP, sulfonylurea, and phosphoinositide binding sites, and interact with L0, an SUR cytoplasmic loop. Inputs from these elements link to the pore via the interfacial helix, which forms an elbow with the outer pore helix. Mutations that destabilize the interfacial helix increase channel activity, reduce sensitivity to inhibitory ATP and channel inhibitors, glibenclamide and repaglinide, and cause neonatal diabetes.**

We compared Kir6.2/SUR1 channels carrying the V59G substitution, a cause of the developmental delay, epilepsy, and neonatal diabetes syndrome, with a V59A substitution and the analogous KCNJ8 I60G mutation in the related Kir6.1 subunit from neonatal diabetes syndrome, developmental delay, epilepsy, and neonatal diabetes syndrome. We found that V59G and V59A channels are either not, or poorly, stimulated by phophatidylinositol 4,5-bisphosphate with neomycin and polylysine is reduced in V59A, and abolished in V59G channels. Stimulation by SUR1 is intact, and increasing the concentration of inhibitory ATP restores the sensitivity of Val-59-substituted channels to glibenclamide. The I60G channels, strongly dependent on SUR1, were either not, or poorly, stimulated by phosphatidylinositol 4,5-bisphosphate and phosphatidylcholine, and did not respond to neomycin and polylysine. The results suggest that the interfacial helix dynamically links inhibitory inputs from the Kir N terminus to the gate and that sulfonylureas stabilize an inhibitory configuration.

ATP-sensitive K<sup>+</sup> channels (K<sub>ATP</sub> channels)<sup>2</sup> consist of four pore-forming subunits Kir6.x and four sulfonylurea receptor (SURx) subunits, which are members of the ATP-binding cassette (ABC) protein superfamily (1). Adenine nucleotides have a balanced action on K<sub>ATP</sub> channels: Mg<sup>2+</sup>-independent nucleotide binding to Kir6.x cloes the channel (2–4), whereas MgATP binding to, or MgATP hydrolysis by, SURx stimulates channel openings (5–9). These results obtained with recombinant systems give an explanation for the older observation that K<sub>ATP</sub> channels are sensitive to the ratio of ADP to ATP (10, 11). There are several subtypes of K<sub>ATP</sub> channels (12) that subserve important functions in many tissues (13). The Kir6.2/SUR1 neuroendocrine channel in pancreatic β-cells couples insulin secretion to the plasma glucose level. K<sub>ATP</sub> channels determine the β-cell membrane potential and changes in the levels of ATP and ADP induced by changes in glucose metabolism determine channel activity. Antidiabetic sulfonylureas such as glibenclamide (GBC) bind to SUR1 reducing its stimulatory action on the pore thus inducing channel closure that prompts insulin secretion (14, 15).

Mutations in either subunit can alter the balanced action of adenine nucleotides on K<sub>ATP</sub> channels and result in disorders of insulin secretion (16, 17). Loss of channel function is a cause of hyperinsulinemic hypoglycemia (reviewed in Ref. 18), whereas mutations that increase channel activity are one cause of neonatal diabetes (reviewed in Ref. 17). Mutations in SUR1 have been identified that produce more active channels via increased Mg-nucleotide-dependent stimulation of the pore (6, 19, 20), reviewed in Ref. 17). Mutations in Kir6.2 have been identified in which the ability of ATP to close the channel is reduced due to a decreased affinity for inhibitory ATP or to an increased stability of the open state in the absence of ATP (16, 21, 22). In patients carrying one copy of these “gain of function” mutations the balanced action of adenine nucleotides is altered and the resulting increase in channel activity leads to β-cell hyperpolarization and the decrease in insulin secretion that causes (transient or permanent) neonatal diabetes. K<sub>ATP</sub> channel subunits are found in neurons (Kir6.2 with SUR1), in striated muscle (Kir6.2 with SUR2A), and in some smooth muscle (Kir6.1 with SUR2B). Some Kir6.2 mutations result in hyperactive channels that produce more severe syndromic phenotypes that include muscle weakness, developmental delay, epilepsy, and neonatal diabetes, termed the DEND syndrome (16, 21, 23–25).

One DEND mutation, V59G, is in the slide or interfacial helix of Kir6.2 (21), an amphipathic stretch of 13 amino acids (resi-
dues 54–66) that lies at the membrane-cytosol interface and is assumed to be important in the mechanics of channel gating (26–28). In intact Xenopus oocytes, homozygous V59G channels are essentially open (29) and in isolated patches, 3 mM MgATP produces only a 10% block; in addition, the sensitivity of heterozygous channels to tolbutamide is strongly reduced (29, 30). In experiments at the single channel level in the absence of ATP, homozygous V59G channels exhibit a high open probability ($P_O = 0.83$ versus 0.53 for the wild type (29)). The reduced sensitivity of V59G channels to inhibition by ATP and tolbutamide has been attributed to the higher open probability, because both compounds stabilize a long lived interburst closed state (31–34). In addition, the V59G substitution reduces the surface expression of the mutant channel to ~20% of wild type (35). Two other substitutions of Val-59 occur, V59M (21) and V59A, which produce an “intermediate” DEND phenotype. The V59M channel has been extensively characterized (22, 29, 30), and the properties of the recently identified V59A channel have not been established.

Homology models imply the interfacial helix could serve to transmit conformational changes from the regulatory subunit to the outer transmembrane (M1) helices of Kir6.1 or 6.2 pores and thus affect gating. The nature of these conformational changes is uncertain, but the first N-terminal residues of Kir6.2 are known to be important for transitions from the open to the long lived closed state. Deletions of 5–35 residues from Kir6.1 and Kir6.2 produce highly active channels, $P_O > 0.9$ (36, 37), with reduced sensitivity to sulfonylureas and ATP (3, 33, 37, 39) similar to those seen in the V59G channels. We suggest that the N terminus of Kir6.x is an inhibitory element that interacts with parts of SUR1, specifically with the L0 linker, to facilitate the transition to the closed state and thus to restrict channel openings (39). The observation that soluble, Kir N-terminal-like peptides can reduce the $P_O$ of ΔNKir6.1/SUR1 and ΔNKir6.2/SUR1 channels supports this idea (36). The interfacial helix is the physical connection between the proximal N terminus and the outer M1 helix. We propose that substitutions (e.g. glycine, methionine, and alanine) for valine at position 59 can introduce flexibility into the V59G helix that impairs the inhibitory action of the proximal N terminus.

To support this hypothesis, we sought to characterize the V59G mutation further, compare it with a V59A substitution and the analogous substitution, I60G, in Kir6.1. The V59A, and I60G mutations were introduced into Kir6.2 and Kir6.1, respectively, using the QuikChange II XL site-directed mutagenesis kit (Stratagene). The mutations were verified by sequencing the relevant DNA region (V59G) or the whole coding region (I60G and V59A).

HEK 293 cells were cultured in minimum essential medium containing glutamine and supplemented with 10% fetal bovine serum and 20 μg/ml gentamycin as described (45). Cells were transfected with wild-type or mutant Kir6.x and SUR1 at a molar ratio of 1:1 using Lipofectamine 2000 and Opti-MEM (Invitrogen) according to the manufacturer’s instructions (46). The pEGFP-C1 vector (Clontech, Palo Alto, CA), encoding green fluorescent protein, was added for identification of transfected cells.

**Patch Clamp Experiments**—Patch clamp experiments in the whole cell configuration were performed at 37 °C as described by Russ et al. (47). The bath was filled with (in mM): NaCl, 142; KCl, 2.8; MgCl$_2$, 1; CaCl$_2$, 1; d (+)-glucose, 11; HEPES, 10; pH 7.4. Patch pipettes were filled with (in mM) potassium glutamate, 132; NaCl, 10; MgCl$_2$, 2; HEPES, 10; EGTA, 1; Na$_2$ATP, 1; and Na$_2$GDP, 0.3 at pH 7.2 such that [Mg$^{2+}$]$_{free}$ was ~0.85 mM and that there was a balance between inhibition and activation. Pipettes had a resistance of 3–5 MΩ. Cells were clamped at ~60 mV. To determine the reversal potential of the currents, square pulses, 0.5-s duration, ranging from ~110 to +10 mV in 20-mV steps were applied every 12 s. Recordings in which the reversal potential deviated from ~90 mV were rejected.

For experiments in the cell-attached and inside-out configuration, pipettes with a larger diameter were used (resistance of 1.0–1.5 MΩ). Experiments were performed at 22 °C. Pipette and bath were filled with a high K$^+$-Ringer solution containing (in mM) KCl, 142; NaCl, 2.8; MgCl$_2$, 1; CaCl$_2$, 1; d (+)-glucose, 11; HEPES, 10; titrated to pH 7.4 with NaOH. In the inside-out mode and after patch excision, the pipette was moved in front of a pipette filled with a high K$^+$ buffer containing (in mM) KCl, 142; MgCl$_2$, 0.7–30.7 (according to the nucleotides added); d (+)-glucose, 11; Na$_2$ATP, 0–30; EGTA, 0.1 (0 when adding Ba$^{2+}$); HEPES, 10; titrated to pH 7.2 with NaOH at 22 °C and containing the channel modulators of interest. For experiments in Mg$^{2+}$-free buffer, MgCl$_2$ was omitted and EGTA was
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replaced by 1 mM EDTA. In a series of experiments using high ATP concentrations (10 and 30 mM), NaCl was added to the bath and pipe solutions with lower ATP concentration to minimize differences in osmolarity. Patches were generally clamped at $-50 \text{ mV}$ except for examination of the Ba$^{2+}$-induced block in the inside-out configuration. Ba$^{2+}$ was applied to the inside of the patch produced only a slight block at $-50 \text{ mV}$ (because it was flushed out of the pore by the inward K$^+$ current at negative voltage), whereas at $+50 \text{ mV}$ a complete block was achieved (as Ba$^{2+}$ was dragged into the pore by the outward current). Some inhibitors (e.g. neomycin) were tested at both $-50$ and $+50 \text{ mV}$. In experiments with the V59G/SUR1 channel in the inside-out configuration and at symmetrical high K$^+$ solution, the leak current was determined as the outward current remaining in the presence of 1 mM Ba$^{2+}$ at $+50 \text{ mV}$, and it was assumed that, at $-50 \text{ mV}$, the leak current was of the same magnitude (with inverted sign), thus determining the zero current level. Data were filtered at 0.2 kHz and sampled at 1 kHz.

The open probability ($P_o$) of the V59A channel was determined in the inside-out configuration using Sylgard-coated pipettes. Recordings from patches with one channel were filtered at 2.5 kHz and sampled at 5 kHz. Amplitude histograms were generated using PulseTools (Heka, Lambrecht, Germany) and analyzed by fitting a superposition of 2 Gaussian distributions to the data.

$[^3H]GBC$ Competition Experiments—Binding experiments were performed in intact cells at $37 \text{ °C}$ as described by Hambrock et al. (45) using an incubation buffer containing (in mM): NaCl, 129; KCl, 5; MgCl$_2$, 1.2; CaCl$_2$, 1.25; D(-)-glucose, 11; NaHCO$_3$, 5; HEPES, 10 at pH 7.4. $[^3H]GBC$ was 1–2 nM; nonspecific binding was determined in the presence of 100 nM GBC and was $\sim 10\%$ of total binding.

Data Analysis and Statistics—Channel inhibition and equilibrium binding curves were analyzed according to the Hill equation,

$$y = 100 - \frac{A}{(1 + 10^{n(pX - IC_{50})})}$$

as described before (45) with $y$ denoting the current or total binding, $A$ the maximum inhibition (amplitude or extent of specific binding), $n_H$, the Hill coefficient, and $x$ the inhibitor concentration with $pX = -\log X$ and $pIC_{50} = -\log IC_{50}$. In the text, the IC$_{50}$ values with their 95% confidence interval are given. IC$_{50}$ values are lognormally distributed (48); therefore the corresponding pIC$_{50}$ values were compared by using the Student $t$-test after the data had passed the normality and equal variance tests using the program SigmaStat 3.1 (SPSS Science, Chicago, IL).

Materials—The reagents and media used for cell culture and transfection were from Invitrogen, the other chemicals, including nucleotides, were from Sigma. Glibenclamide, tolbutamide, and diazoxide were purchased from Sigma; repaglinide was a kind gift from Novo Nordisk (Bagsvaerd, Denmark). The K$_{ATP}$ channel modulators were dissolved in DMSO/ethanol (50/50, v/v) and further diluted with the same solvent or with incubation buffer (final solvent concentration in the assays, $<1\%$). $[^3H]GBC$ (specific activity, 1.85 TBq/mmol) was purchased from Perkin-Elmer Life Sciences. Poly-d-lysine-HBr (mean molecular weight 41,400; chain length $\sim 200$) was from Sigma.

RESULTS

Structure Prediction—Glycine has a lower helix propensity than valine (49), and two, Predahtor and nnpredict, out of five protein secondary structure programs predicted that the V59G substitution will destabilize and thus break the interfacial helix. The PSIPRED and PhD programs predicted no structural change with the V59G mutation; IPred did not recognize the helix structure. The same structural predictions were obtained for the I60G substitution in Kir6.1. Alanine and methionine have a greater helix propensity on the Chou-Fasman scale (49), and two, Predator and nnpredict, out of five protein secondary structure programs predicted that the V59G substitution will destabilize and thus break the interfacial helix.

Basic Observations on Kir6.2 Channels—Fig. 1 shows basic characteristics of the wild-type and Val-59-substituted channels determined in the whole cell configuration. After breaking
into a cell expressing wild-type channels, a current developed
during cell dialysis with a nucleotide containing activating solu-
tion (Fig. 1a). The current was totally inhibited by gliben-
clamide (GBC, 0.1 μM), indicating it goes through K$_{ATP}$ chan-
nels. In contrast, in cells expressing the Val-59-substituted
channels, a current was present immediately upon breaking
into the cell indicating channels were open prior to dialysis (Fig.
1, b and c). In agreement with their reported reduced sensitivity
to sulfonylureas (29) the V59G currents were not affected by
GBC (1 μM) (Fig. 1b; n = 12). The V59A channels display an
intermediate sensitivity to 1 μM glibenclamide, ~40% inhibition.
Both of the Val-59-substituted channels are blocked by a high
concentration of Ba$^{2+}$ (1 mM). Fig. 2 shows there is no
significant difference in Ba$^{2+}$ sensitivity between the wild-type
and V59G channels.

Inhibition by ATP, GBC, and Their Combination—As shown
in Fig. 1b, 1 μM GBC had no significant effect on the V59G
channel when cells were dialyzed with a standard activating
solution containing 1 mM ATP and 0.3 mM GDP. Under these
conditions MgATP will inhibit channel activity via binding to
Kir and stimulate activity via binding and hydrolysis on SUR1
(2, 3, 6). The Kir inhibitory nucleotide-binding site is adenine-
selective (34, 50), thus MgGDP primarily stimulates channel
activity. Dialysis with 10 mM MgATP alters the nucleotide bal-
ance by adding an inhibitory component, and now 1 μM GBC
inhibits the whole cell V59G currents by 20 ± 4% (n = 4; data
not shown). This suggested that high concentrations of MgATP
could “potentiate” channel inhibition by GBC. We used the
inside-out membrane configuration to analyze this effect fur-
thier (Fig. 3). Fig. 3a shows that the combined inhibitory and stimula-
tory actions of 10 mM MgATP produced a small, ~7%, inhibition. In
the absence of nucleotides, 1 μM GBC was inactive, but the co-appli-
cation of 10 mM MgATP induced a more marked, ~24%, inhibition.
Under these conditions SUR1, whose affinity for GBC is >10$^9$ M$^{-1}$,
is saturated with the sulfonylurea, which strongly inhibits its Mg-
nucleotide-dependent stimulatory action on Kir6.2. Thus the apparent
“potentiation” of GBC by 10 mM MgATP reflects the combined
inhibitory actions of ATP and GBC after disruption of nucleotide stim-
ulation by the sulfonylurea. Fig. 3b provides support for this interpreta-
tion. MgATP (30 mM) has balanced inhibitory versus stimulatory effects
on channel activity via binding to Kir2.2 and SUR1, respectively. Lim-
iting the stimulatory action of SUR1 with 0.01 μM GBC unmasks the
inhibitory effect of 30 mM MgATP producing ~70% inhibition. The application of additional GBC
(0.1–1 μM) did not result in further inhibition (n = 3–5).
MgATP inhibition curves for the Val-59-substituted channels
in the presence and absence of GBC (1 μM) are presented in Fig.
4 (upper panels); the resulting IC$_{50}$ values are listed in Table 1.
MgATP alone weakly inhibited V59G channels, but blocking
the stimulatory action of SUR1 with 1 μM GBC shifted the inhibi-
tion curve ~10× toward the left (Fig. 4). The V59A chan-
nels were ~90 times more sensitive to inhibition by MgATP
than the V59G channels. Similarly, blocking SUR1 stimulation
with GBC potentiated inhibition of V59A channels by MgATP
~6-fold (Fig. 4, upper panel, and Table 1).

In the absence of Mg$^{2+}$, ATP inhibits channel activity by
binding to Kir6.2, but the Mg$^{2+}$-nucleotide dependent stimula-
tory action of SUR1 is absent. Fig. 3c shows that ATP$^{4-}$ (3 mM)
inhibited K$_{ATP}$ currents by 25%, and the addition of GBC
(0.01 μM) increased this inhibition to ~60%. Higher concentra-
tions of GBC (up to 1 μM) had no further effect. Thus in the
absence of the Mg$^{2+}$-dependent stimulatory action of SUR1,
ATP, and GBC both stabilize the interburst closed state. GBC (1 μM)
alone did not affect the V59G channel, inhibited the V59A
channels ~15% and the wild-type channels ~70% (Table 2), the
maximum inhibition attainable in inside-out patches. The ATP
inhibition curves in the absence of Mg$^{2+}$ are shown in Fig. 4
(lower panels). ATP inhibited V59G channels with an IC$_{50}$ of
~21 mM GBC (1 μM) shifted the ATP inhibition curve to ~7×
lower concentrations (Table 1). The V59A channels were ~65-
times more sensitive to inhibition by ATP$^{4-}$ than the V59G
channels; blocking SUR1 stimulation with GBC potentiated the

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![Graph](image-url)
inhibitory action of ATP$^4$− -3-fold (Fig. 4, lower panel, and Table 1).

To examine further the effect of an increased open probability ($P_O$) on the sensitivity of the channel toward GBC, we used an engineered subunit lacking N-terminal amino acids 2–10, Kir6.2ΔN10. Deletion of the N terminus impairs the transition to the interburst closed state thus increasing the open probability, $P_O$ (33, 37, 38). Table 2 shows that this mutant has an impaired sensitivity to GBC (1 μM), which resembles that observed in the Val-59-substituted channels.

Lipids and Polycations—The effect of channel-activating lipids and inhibitory polycations on Kir6.2/SUR1 channels was studied in the inside-out configuration; sample traces for the wild-type versus V59G channels are shown in Fig. 5; the results are summarized in Table 2. Application of PIP$_2$ (10 μM) or oleoyl-CoA (1 μM) increased the current through wild-type channels by 900 and 390% (n = 13) and 300 and 90% (n = 11, data not shown), respectively. The effect of the lipids was greater when applied after considerable rundown had occurred. Activation by PIP$_2$ was poorly reversible upon washout, whereas the effect of oleoyl-CoA was rapidly reversed. In contrast, PIP$_2$ and oleoyl-CoA were unable to further increase the $P_{O_{\text{max}}}$ of V59G channels (n = 4–7) and had only small stimulatory effects on V59A channels (Table 2). To determine the contribution of PIP$_2$ to the elevated $P_{O_{\text{max}}}$ of the Val-59-substituted channels, we assessed the effects of agents that sequester phosphoinositides. The polycations, neomycin, gentamycin, and polylysine, inhibited wild-type channels with IC$_{50}$ values of 16 and 82 μM and 1 g/ml, respectively. Inhibition of wild-type channels by neomycin and gentamycin was reversible upon washout; inhibition by polylysine was not. In contrast, the Val-59-substituted channels were only weakly affected by these polycations. Inhibition at 50 mV was only slightly stronger. The inhibitory actions of neomycin on the V59A and Kir6.2ΔN10Kir6.2 channels were comparably attenuated (Table 2).

V59G Channels Have a Reduced Efficacy, but Not Affinity for the KATP Channel Antagonist, Repaglinide—Previous work showed that coexpression of wild-type Kir6.2 increased the affinity of SUR1 for glibenclamide ~3-fold and for repaglinide ~130- to 200-fold (51, 52). It was therefore of interest to determine whether the V59G channels had an altered affinity for repaglinide. Binding experiments on intact cells gave a $K_i$ value of 0.70 (0.55 and 0.87) nM (n = 3) for the V59G channels comparable to that determined.
for wild-type channels (0.72 (0.60 and 0.87) nM (51)). Functionally, repaglinide had an effect similar to GBC. In experiments similar to those in Fig. 3a repaglinide (1 μM) did not inhibit the mutant channel by itself but increased the inhibition by MgATP (10 mM) from 5 ± 2 to 50 ± 7% (n = 4, data not shown).

Response of Kir6.1/SUR1 Channels to Nucleotides and Sulfonylureas—Fig. 6 compares the responses of cells expressing wild-type versus I60G/SUR1 channels to stimulation with 1 mM MgATP/0.3 mM MgGDP. Similar to Kir6.2/SUR1 channels, wild-type Kir6.1/SUR1 channels activated upon dialysis, whereas the Kir6.1I60G/SUR1 channels were open immediately upon seal formation and showed little rundown (n = 15) thus resembling the V59G channels. An immediate, stable current was also obtained when cells expressing the I60G channels were dialyzed with 10 mM MgATP indicating that 10 mM MgATP did not close the mutant channel (n = 5). Dialysis with Mg2+ - or nucleotide-free solutions led to a steady loss of current over 2–10 min indicating that the I60G channel required activation to remain in the open state (n = 12). GBC (10 mM) inhibited both the wild-type and I60G channels ≥90% (Fig. 6). This inhibition of the I60G channels is in sharp contrast to the V59G Kir6.2 channels (Fig. 1b), which were insensitive to GBC up to 1 μM under equivalent activating conditions.

The inhibition of SUR1 containing channels by glibenclamide is essentially irreversible on the time scale of electrophysiological experiments. Tolbutamide, which inhibits the pancreatic KATP channel with ~1000-fold lower potency and is rapidly reversible, was also tested. In the whole cell configuration, wild-type Kir6.1/SUR1 channels were completely and reversibly inhibited by tolbutamide (100 μM), whereas the I60G channels were 89 ± 6 and 91 ± 4% inhibited by 100 and 300 μM tolbutamide (n = 4 and 8), respectively. Assuming complete inhibition, an IC50 value of ~10 μM was estimated for the I60G channel.

The experiments in the whole cell configuration (Fig. 6) suggest that, in the cellular environment, wild-type Kir6.1 channels are essentially closed, whereas the I60G channels are open. These observations were confirmed using the cell-attached configuration of the patch clamp technique. Fig. 7a shows activity was low when wild-type Kir6.1 channels are exposed to cellular nucleotide levels. Application of 100 μM diazoxide stimulates the activity of channels with bursting behavior characteristic of KATP channels and 1.8 pA currents expected for a single channel conductance of 36 pS in symmetrical high K+ solution (41) at a patch potential of −50 mV (n = 13). The diazoxide-induced activity of wild-type Kir6.1 channels was blocked reversibly by tolbutamide (300 μM). As shown in Fig. 7b, the I60G channels were spontaneously active in on-cell patches and exhibited transitions between long lived states that differed by ~1.8 pA. Tolbutamide (300 μM) reversibly inhibited the I60G channels (Fig. 7b), whereas diazoxide had no effect (n = 9). Diazoxide and tolbutamide had no effect on non-transported cells (n = 7).

### DISCUSSION

**Structural Hypothesis**—We compared Val-59-substituted Kir6.2/SUR1 and analogous I60G Kir6.1/SUR1 channels with their respective wild-type channels. The mutations are located in the middle of a 13-amino acid helix, termed the interfacial or slide helix, that lies at the membrane-cytosol interface and forms an elbow with the outer transmembrane helix, M1, via a short loop (26, 27). In Kir6.2, the interfacial helix is flanked by basic residues, Arg-54 and Lys-67, proposed to be part of the PIP2 binding site (53, 54) and is C-terminal of Arg-50 and Arg-54 proposed to be part of the inhibitory adenine nucleotide binding site (39, 53, 55). The interfacial helix is adjacent to a peptide segment, residues 37–44, which specifies major gating characteristic of KATP channels and 1.8 pA currents expected for a single channel conductance of 36 pS in symmetrical high K+ solution (41) at a patch potential of −50 mV (n = 13). The diazoxide-induced activity of wild-type Kir6.1 channels was blocked reversibly by tolbutamide (300 μM). As shown in Fig. 7b, the I60G channels were spontaneously active in on-cell patches and exhibited transitions between long lived states that differed by ~1.8 pA. Tolbutamide (300 μM) reversibly inhibited the I60G channels (Fig. 7b), whereas diazoxide had no effect (n = 9). Diazoxide and tolbutamide had no effect on non-transported cells (n = 7).

### TABLE 1

**Potency of ATP inhibition of V59G and V59A currents**

Effects of Mg2+ and GBC (1 μM). IC50 values (followed by the 95% confidence interval) were determined as described in Fig. 4. Statistical tests were performed with the respective pk values (which are normally distributed (49)).

| Conditions | V59G | V59A |
|------------|------|------|
| Mg2+ (0.7 mM) | GBC (1 μM) | IC50 | IC50 | nH |
| + | − | − | 200 | 2.3 (0.9, 5.9) | 0.47 ± 0.13 |
| + | + | 20 (10, 38) | 0.36 (0.28, 0.48) | 0.85 ± 0.10 |
| − | + | 21 (15, 31) | 0.32 (0.21, 0.49) | 1.3 ± 0.2 |
| − | − | 3.1 (1, 9, 51) | 0.11 (0.077, 0.15) | 1.3 ± 0.2 |

* nH is the Hill coefficient; for V59G nH was set to 1.0.
* a Value uncertain (cf. Fig. 4).
* b Different from values in the presence of either Mg2+ or GBC (but not both), p < 0.05.
* c Different from the corresponding value in the presence of Mg2+, p < 0.05.

### TABLE 2

**Properties of Kir6.2 wild type, V59G, V59A, and ΔN10 channels formed with SUR1**

Experiments in inside/outside patches – Mg2+; number of experiments are in parentheses.

| Kir6.2 | P0 | GBC (1 μM) | PIP2 (10 μM) Ihyp/4Icontest | Neomycin |
|--------|----|------------|-----------------------------|---------|
| Wild type | 0.32 ± 0.10 (5)* | 71 ± 10 (4) | 900 ± 390 (12) | 57 ± 5 (9) |
| V59G | 0.83 ± 0.01 (8)* | 0 (10) | 0 (4) | 3 ± 2 (5) |
| V59A | 0.86 ± 0.01 (4) | 15 ± 8 (7) | 5 ± 3 (5) | 17 ± 6 (6) |
| ΔN10 | 0.61 ± 0.08 (4) | 9 ± 5 (12) | 270 ± 40 (4) | 17 ± 8 (10) |

* P0 is the open probability of single channels in the absence of nucleotides.
* a Data from Reimann et al. (38) in Xenopus oocytes.
* b Data from Proks et al. (29) in Xenopus oocytes.
the integrity of the interfacial helix. Structure prediction programs suggest that substitution of a glycine for valine at position 59 will introduce a break, a short extended sheet, in the interfacial helix that would introduce flexibility and impair signal transmission. Substitution of methionine or alanine for valine is not predicted to have as drastic an effect on helix structure and does perturb signal transduction to a lesser, albeit physiologically significant degree because both mutations produce a severe phenotype.

There is strong evidence for the view that the proximal N termini of Kir6.2 (and Kir6.1) are “inhibitory” segments critical for the transition of these channels to the long lived interburst closed state. This idea is based on the observations that deletion of the Kir N terminus increases the channel \( P_o \) by impairing the transition to the closed state (3, 33, 38) and that a synthetic Kir6.2 N-terminal-like peptide can reduce the \( P_o \) of Kir6.2ΔN32/SUR1 channels (36). In addition, application of the synthetic Kir6.2 N-terminal-like peptide to intact SUR1/Kir6.2 channels increases their \( P_o \), presumably by displacing the endogenous N terminus (36). We hypothesize that an increased flexibility of the interfacial helix would impair transmission of the inhibitory N-terminal signal and thus impede transitions to the interburst closed state. This will reduce the potency of inhibitory ATP (and GBC) and thus produce more active channels that, interestingly, have a reduced requirement for PIP2 to maintain their activity.

**Sensitivity to ATP**—Experiments in the inside-out configuration showed that in the absence of Mg\(^{2+}\), the V59G channel had an \(~4000\)-fold lower sensitivity toward inhibition by ATP\(^{4-}\) than the wild-type (21 mM versus 5 \( \mu \)M). For comparison, deletion of up to 44 N-terminal amino acids from Kir6.2 led to a 10- to 20-fold reduction in the sensitivity to inhibitory ATP (3, 33, 38). At least two explanations could rationalize this difference. The V59G mutation could reduce the affinity for inhibitory ATP more strongly than deletion of the N terminus in the \( \Delta N \)Kir channels. Alternatively, signal transduction may be more seriously impaired by the V59G mutation. ATP is suggested to stabilize the interburst closed state, and thus the reduced sensitivity to inhibitory ATP has been hypothesized to reflect the reduced time the V59G and \( \Delta N \)Kir channels spend in the closed state. Both of the Val-59 substitutions and N-terminal truncation produce channels with \( P_o \) values > 0.8, but the Val-59-substituted channels are far less sensitive to inhibition by ATP. We suggest that both types of “mutation” affect the same inhibitory linkage, but in somewhat different ways. The results are consistent with a “sequential transduction model” in which the interfacial helix acts as the final common element that transduces multiple signals to the pore, including inputs from the proximal N-terminal segment, L0, and the ATP and PIP2 binding sites. The increase in \( P_o \) attributable to N-terminal truncation reflects removal of one inhibitory input, whereas destabilization of the interfacial helix by the Val-59 substitutions weakens or abolishes multiple inputs, including that from the N terminus. Destabilization is greatest for substitution with glycine, which can adopt the widest range of psi-phi angles. We presume that both the Val-59-substituted and \( \Delta N \)Kir subunits retain ATP binding, perhaps with reduced affinity. We suggest that the inhibitory effect of

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**FIGURE 5.** Traces showing the effects of lipids and polycations on wild-type and V59G \( K_{ATP} \) channels at \(~50 \text{ mV}. The zero current level is indicated by the dotted line. a, PIP2 (10 \( \mu \)M); b, neomycin (30 and 1000 \( \mu \)M); and c, polylysine (100 \( \mu \)g/ml).

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ATP on a ΔNKir channel is greater, only a 10- to 20-fold reduction, because the interfacial helices are intact and able to transduce the effect of ATP binding, whereas the link is destabilized in the V59A and V59M channels and largely absent in V59G channels.

In the presence of Mg2+, inhibition of the Val-59-substituted channels by MgATP was ~7–10 times weaker than for ATP4−, consistent with effective Mg-nucleotide-dependent stimulation via SUR1. Assuming that MgATP and ATP interact with the Val-59-substituted channels with equal affinity, the results indicate the “pathway” for the stimulatory action of MgATP via SUR1 is intact in the Val-59-substituted channels. Babenko (6) has reported a comparable (~10-fold) MgATP-dependent net stimulation of wild-type channels. This implies the increased mutant channel activity is primarily due to reduced nucleotide inhibition (see however, Ref. 30, which suggests the stimulatory action of MgATP via SUR1 is enhanced in Val-59-substituted channels).

Information on the Kir6.1I60G/SUR1 channels is more limited and their reduced expression impeded experiments in the inside-out configuration. Whole cell and cell-attached recordings show that the I60G channels are more active than wild-type in intact cells (Fig. 7). This suggests that under physiologic conditions the I60G channels either have a reduced sensitivity to inhibitory ATP versus wild-type or are more efficiently activated by MgATP. We did not observe I60G channel activity in the absence of Mg2+ and activating nucleotides consistent with earlier studies on the Kir6.1/SUR1 wild-type (34, 40, 42, 43), whereas both the Val-59-substituted and wild-type Kir6.2 channels are open in the absence of activating nucleotides (e.g. Figs. 3 and 5). Kondo et al. (56) have shown that amino acids 37–44 are the major determinant of the requirement for nucleotide stimulation in Kir6.1 channels, while a C-terminal sequence, 243–248, has a lesser role. The mechanism by which these two short segments in Kir6.1 prevent opening of the channel in the absence of SUR activation is unknown. We note that deletion of the Kir6.1 N terminus, ΔN33Kir6.1, which would remove the putative inhibitory segment produces ΔN33Kir6.1/SUR1 channels that are spontaneously active in the absence of activating nucleotides and whose P_o is reduced by the Kir6.2 synthetic peptide (36). One interpretation of these results is that the putative N-terminal inhibitory segment of Kir6.1 is more firmly tethered to its binding site than its Kir6.2
counterpart and thus exhibits an increased dependence on SUR activation for activity. In support of this idea, we note that, although the application of the Kir6.2 synthetic peptide to intact Kir6.1/SUR1 channels does increase their activity, the effect is smaller than that observed with intact Kir6.2/SUR1 channels (36).

**Sensitivity to GBC**—GBC (1 μM) was without effect on V59G channel activity under standard activating conditions (1 mM MgATP, 0.3 mM MgGDP) and had a markedly reduced inhibitory action on the V59A channels. Interestingly, although the GBC binding site is composed of parts of SUR1 and the N terminus of Kir6.2 (58, 59), and deletion of the Kir6.2 N terminus reduces sensitivity to sulfonylureas (33, 37, 38), the reduced inhibition of Val-59-substituted channels is not the result of altered GBC or repaglinide binding to SUR1. With a $K_d$ value of 0.5 nM (51), at 1 μM GBC, binding to both wild-type and mutant channels was 99.95% saturated. The reduced effect of GBC on the Val-59-substituted channels implies that the mechanisms by which sulfonylurea binding to SUR1 closes the channel are markedly less effective when the inhibitory transduction pathway is disrupted (Fig. 1). GBC limits the activating effect of Mg-nucleotides; however the hyper-activation observed in the Val-59-substituted channels also requires that the normal counterbalancing nucleotide inhibition is reduced. GBC stabilizes long lived closed states, but these are reduced dramatically, because the inhibitory linkage is disrupted and nucleotide inhibition is impaired in the Val-59-substituted and ΔNKir6.2 channels. Two observations support this interpretation. First, partially restoring the balance between activation and inhibition by significantly increasing the concentration of ATP confirms that the activation pathway is intact and is inhibited by GBC (Fig. 3 and see “Discussion” below). Second, the I60G channels, whose activity is strongly dependent on Mg-nucleotide-dependent stimulation by SUR1, are nearly as sensitive to GBC as the wild-type (potency difference $\sim 4X$). GBC limits the stimulatory input from SUR1 thereby inducing closure of the I60G channels.

**Effect of ATP and GBC in Combination**—We observed synergistic effects when ATP and GBC were co-applied to Val-59-substituted channels. We suggest these effects reflect the altered balance of inhibitory versus stimulatory actions of nucleotides on $K_{ATP}$ channels and support a role for the Kir N terminus in GBC inhibition. In the inside-out configuration of the patch clamp technique, concentrations of sulfonylureas that saturate SUR1 are reported to reduce the activity of wild-type channels $\sim 50\%$ when tested in the absence of nucleotide activation ($-Mg^{2+}$) and to show an increased efficacy under activating conditions (60, 61). Deletion of 10–20 N-terminal residues from Kir6.2 reduces the potency of ATP$^{4-}$ to $20\times$ (IC$_{50}$ increase from $\sim 5$ to 100 μM) and eliminates sensitivity to a saturating concentration (200 μM) of tolbutamide without affecting the Mg-nucleotide-dependent stimulatory pathway (33, 37, 38). In the absence of stimulation ($-Mg^{2+}$), the V59G mutation increases the IC$_{50}$ for ATP from $\sim 5$ μM to $\sim 20$ mM and 1 μM GBC, ineffective when applied alone, shifts the ATP$^{4-}$ inhibition curve to $\sim 7\times$ lower concentrations (Fig. 4 and Table 1). The V59A results were broadly similar. These observations emphasize the interplay between the proposed inhibitory segments in the Kir N terminus, the interfacial helix, and GBC binding to SUR1. The observations suggest a speculative hypothesis: We assume that the interactions of the Kir N terminus with SUR1 are dynamic, as suggested by experiments with a synthetic N-terminal peptide (36), and we hypothesize that GBC binding to SUR1 stabilizes a non-stimulatory configuration. In this case, deletion of the N terminus, in the ΔNKir6.2 channels, eliminates the inhibitory segments, abolishes GBC inhibition and, as discussed above, impairs inhibition by nucleotides.

Similarly, although the Kir N terminus is intact in the Val-59-substituted channels, destabilizing the interfacial helix impairs dynamic signaling to the gate. The stabilization of an inhibitory conformation of the Kir N terminus by ATP and/or GBC can partially overcome the disruption of the interfacial helix. When Mg$^{2+}$ is present the strong stimulatory action of SUR1 is apparent; the IC$_{50}$ of the V59A channels is increased, $\sim 5$ μM to 2.3 mM, whereas the IC$_{50}$ for the V59G channels can only be estimated at $\sim 200$ mM. The combined effect of ATP inhibition and GBC reducing the stimulatory action of SUR1 and stabilizing an inhibitory conformation of the Kir N terminus shifts the inhibition curve for the V59G channels to $\sim 10$-fold, and the V59A to $\sim 6$-fold, lower concentrations, similar to the effect of ATP$^{4-}$ in the absence of Mg$^{2+}$ and GBC ($\sim 20$ mM for V59G and 0.32 mM for V59A, respectively, Table 1). These quantitative effects are clinically relevant; patients with the V59G mutation are not responsive to sulfonylureas, whereas the V59A patient has responded to GBC.$^4$

The inhibitory potency of GBC was explored using a synergism protocol with the V59G channels (Fig. 3). At high ATP concentrations, in the presence and absence of activation ($\pm$ Mg$^{2+}$), the maximum level of inhibition was reached at $<10$ nM GBC, consistent with an affinity of GBC $\leq 1$ nM. The result is in agreement with the lack of effect of the V59G mutation on GBC binding.

**PIP$_2$ and Polycations**—The V59G channels are totally insensitive to activation by PIP$_2$ (Fig. 5) and oleoyl-CoA. This may simply reflect the fact that they are maximally active. However, the polycations, gentamycin, neomycin, and polylysine, produced only a partial block at high concentrations. The V59A channels showed only a small activation by PIP$_2$ and a markedly reduced sensitivity to neomycin. Similarly, the ΔNKir6.2 channels were activated by PIP$_2$, albeit to a lesser degree than wild-type, and the inhibitory effect of phosphoinositide sequestration by neomycin was reduced (Table 2) consistent with a reduced requirement for PIP$_2$. We cannot eliminate the possibility that alteration of the N termini actually greatly increases the affinity of the Kirs for PIP$_2$, but favor the idea that the affinities are reduced and that the reduction of Val-59-substituted channel activity by polycations does not involve sequestration of PIP$_2$, but rather that they partially block the channel pore at the high concentrations used.

$Ba^{2+}$—The Val-59-substituted channels were completely blocked by Ba$^{2+}$ applied from the outside. Experiments in the

$^4$ L. Philipson, personal communication.
inside-out mode showed there was no difference in the blockade of V59G versus wild-type channels. The site of Ba\(^{2+}\) block is located at the inner side of the selectivity filter (62); the data imply the V59G substitution in the interfacial helix does not affect the Ba\(^{2+}\) site.

In conclusion, we have compared Kir6.x/SUR1 channels with Kir substitutions predicted to increase the flexibility of, or disrupt, the interfacial helix and thus impair signal transduction within the channel. The substituted channels have strongly increased \(P_o\) values in the cellular environment. Disruption of the interfacial helix in the V59G channel effectively uncouples the pore from nucleotide inhibition and phosphoinositide activation, actions that require direct binding to the pore, without affecting the binding of GBC or repaglinide to SUR1. The V59A results are consistent with this substitution partially destabilizing the interfacial helix. Destabilization of the interfacial helix in the V59G channel effectively uncouples the binding of GBC or repaglinide to SUR1. The V59A substitution in the interfacial helix partially destabilizes the interfacial helix, affecting the binding of GBC or repaglinide to SUR1. The V59A substitution affects the binding of GBC or repaglinide to SUR1. The V59A substitution affects the binding of GBC or repaglinide to SUR1. The V59A substitution affects the binding of GBC or repaglinide to SUR1. The V59A substitution affects the binding of GBC or repaglinide to SUR1. The V59A substitution affects the binding of GBC or repaglinide to SUR1.
Slide Helix Mutations in Kir6.x

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