Molecular basis of Kir6.2 mutations associated with neonatal diabetes or neonatal diabetes plus neurological features

Peter Proks*†, Jennifer F. Antcliff*, Jon Lippiat*, Anna L. Gloyn‡§, Andrew T. Hattersley‡, and Frances M. Ashcroft*¶

*University Laboratory of Physiology, Oxford University, Oxford OX1 3PT, United Kingdom; ‡Institute of Biomedical and Clinical Science, Peninsula Medical School, Exeter EX2 5DW, United Kingdom

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Inwardly rectifying potassium channels (Kir channels) control cell membrane K⁺ fluxes and electrical signaling in diverse cell types. Heterozygous mutations in the human Kir6.2 gene (KCNJ11), the pore-forming subunit of the ATP-sensitive (K_ATP) channel, cause permanent neonatal diabetes mellitus (PNDM). For some mutations, PNDM is accompanied by marked developmental delay, muscle weakness, and epilepsy (severe disease). To determine the molecular basis of these different phenotypes, we expressed wild-type or mutant (R201C, Q52R, or V59G) Kir6.2/sulfonylurea receptor 1 channels in Xenopus oocytes. All mutations increased resting whole-cell K_ATP currents by reducing channel inhibition by ATP, but, in the simulated heterozygous state, mutations causing PNDM alone (R201C) produced smaller K_ATP currents and less change in ATP sensitivity than mutations associated with severe disease (Q52R and V59G). This finding suggests that increased K_ATP currents hyperpolarize pancreatic beta cells and impair insulin secretion, whereas larger K_ATP currents are required to influence extrapancreatic cell function. We found that mutations causing PNDM alone impair ATP sensitivity directly (at the binding site), whereas those associated with severe disease act indirectly by biasing the channel conformation toward the open state. The effect of the mutation on ATP sensitivity in the heterozygous state reflects the different contributions of a single subunit in the Kir6.2 tetramer to ATP inhibition and to the energy of the open state. Our results also show that mutations in the slide helix of Kir6.2 (V59G) influence the channel kinetics, providing evidence that this domain is involved in Kir channel gating, and suggest that the efficacy of sulfonylurea therapy in PNDM may vary with genotype.

Materials and Methods

Mutagenesis and RNA Preparation. Human Kir6.2 (GenBank accession no. NM000525) and rat SUR1 (GenBank accession no. L40624) were used in this study. Because human Kir6.2 contains two common polymorphisms, E23K and I377V, we used the most common allele at these residues (i.e., E at position 23 and I at position 377). Site-directed mutagenesis of Kir6.2 was performed with the QuikChange XL system (Stratagene). Wild-type and mutant mRNAs were expressed in Xenopus laevis oocytes as described in ref. 9. Currents were recorded 1–3 days after injection with 0.1 ng of wild-type or mutant Kir6.2 mRNA and ~2 ng of SUR1 mRNA (giving a 1:20 ratio). For each batch of oocytes, all mutations were injected to enable direct comparison of their effects.

Two-Electrode Voltage-Clamp Studies. Whole-cell currents were recorded from intact oocytes in response to voltage steps of ±20 mV from a holding potential of −10 mV, and they were filtered at 1 kHz and digitized at 4 kHz. Oocytes were constantly superfused with a modified HEPES-based solution (133 mm NaCl, 5 mm KCl, 2 mm MgCl₂, 1 mm CaCl₂, 10 mm HEPES; pH 7.4 at 22°C) to maintain a resting membrane potential of −60 mV. Oocytes were held at −100 mV for 30 s before the beginning of each experiment.

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perfused at 20–24°C with solution containing 90 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, and 5 mM Heps, pH 7.4 with KOH. Metabolic inhibition was produced by 3 mM Na-azide. Tolbutamide (0.5 mM) was tested on all oocytes to confirm that the observed current flowed through Kₐ₅P channels.

**Patch-Clamp Studies.** Macroscopic currents were recorded from giant excised inside-out patches by using the patch-clamp technique. Currents were filtered at 0.15 kHz and digitized at 0.5 kHz. The pipette solution contained 140 mM KCl, 1.2 mM MgCl₂, 2.6 mM CaCl₂, and 10 mM Heps, pH 7.4 with KOH. The internal (bath) solution contained 107 mM KCl, 1 mM K₂SO₄, 10 mM EGTA, and 10 mM Heps, pH 7.2 with KOH, and nucleotides as indicated. All experiments were carried out in the absence of Mg²⁺ to prevent the activating effects of nucleotides mediated by SUR1 (3). Rapid exchange of internal solutions was achieved with a sewer pipe system. Experiments were conducted at 20–24°C. The macroscopic slope conductance, Gₛ, was measured with 20 and −100 mV (9). ATP concentration–response curves were fit with the Hill equation:

\[ G = \frac{G_{MAX}}{1 + \left(\frac{[ATP]}{IC_{50}}\right)^h} \]

where \( [ATP] \) is the ATP concentration, \( G_{MAX} \) is the ATP concentration at which inhibition is half maximal, and \( h \) is the slope factor (Hill coefficient). To control for possible rundown, \( Gₛ \) was taken as the mean of the conductance in control solution before and after ATP application.

Single-channel currents were recorded from small inside-out membrane patches at 60 mV, filtered at 5 kHz, and sampled at 20 kHz. Open probability was determined (in the absence of ATP) from current records of \( \approx 1 \)-min duration shortly after patch excision as \( I/I_{N} \), where \( I \) is the mean macroscopic current, \( I_{N} \) is the single-channel current, and \( N \) is the number of channels (taken as the maximum number of open levels). For heterozygous channels (Fig. 1C), the mean \( P_O \) represents the average of all types of channels (\( P_O \)).

**Data Analysis of Heterozygous Channels.** For analysis of heterozygous channels, we assumed independent mixing of wild-type and mutant subunits. Thus, the relative numbers of Kₐ₅P channels with different subunit compositions (Fig. 1C) were expected to follow the binomial distribution as described in refs. 2 and 10. The values of IC₅₀WT and Pₒ,W (the intrinsic open probability; i.e., the open probability in the unliganded state) for the heterozygous channel population can then be obtained from the following equations:

\[ \frac{16}{(1 + [ATP]/IC_{50,WT})} = \sum_{i=0}^{4} \frac{4!}{(4-i)! \cdot i!} \cdot (1 + [ATP]/IC_{50,j}) \]  

and

\[ 16 \cdot P_{O,H} = \sum_{i=0}^{4} \frac{4!}{(4-i)! \cdot i!} \cdot P_{O,j} \]

where the index, \( i \), denotes the number of mutant subunits (0 < \( i < 4 \)) and IC₅₀,j and Pₒ,j stand for the IC₅₀ and Pₒ of channels containing \( i \) mutant subunits. Assuming that the energy of the channel open state, \( O \), is simply the sum of the contributions of the four individual subunits in the channel (\( G_{O,j} \)):

\[ G_O = \sum_{j=1}^{4} G_{O,j} \]  

The values of IC₅₀,j and Pₒ,j were estimated from the following equations (for further details see Supporting Text, which is published as supporting information on the PNAS web site):

\[ P_{O,j} = P_{MAX}/(1 + \lambda^j A_S) \]  

and

\[ IC_{50,j} = IC_{50,WT} P_{O,WT}/(\lambda^j P_{O,j}) \]

where \( P_{MAX} \) denotes the maximum (intraburst) open probability, \( A_S \) denotes the contribution of interburst closures to the channel open probability, and the parameter \( \lambda \) is the difference in the contribution of the individual channel subunit \( j (1 < j < 4) \) to the energy of the open state (\( G_{O,j} \)) between the mutant (\( G_{O,M,j} \)) and the wild-type (\( G_{O,W,j} \)) channel:

\[ \lambda = \exp((G_{O,M,j} - G_{O,W,j})/RT) \]

The values of \( \lambda \) (0.62), \( P_{MAX} \) (0.86), and \( A_S \) (0.62) were calculated from Eqs. 4–6 by using measured parameters for wild-type and homomeric mutant channels; the values of IC₅₀ and Pₒ for all individual species of heteromeric channels were determined from Eqs. 4 and 5 and for the heterozygous channel population from Eqs. 1 and 2.

**Results and Discussion**

To examine the functional differences between the two classes of mutations, we expressed wild-type or mutant human Kir6.2, together with SUR1, in Xenopus oocytes (9). We first compared the effect of mutations on the properties of homomeric channels composed of a single type of Kir6.2 subunit. We then simulated heterozygosity by expressing a 1:1 mixture of wild-type and mutant Kir6.2 mRNAs with SUR1, which is expected to result in a mixed population of channels containing different ratios of wild-type and mutant subunits (Fig. 1C). We refer to this population as heterozygous channels.

Unlike Wild-Type Channels, Mutant Kₐ₅P Channels Are Not Closed by Resting ATP Levels. When expressed in Xenopus oocytes, wild-type Kₐ₅P channels are normally closed but they are activated by metabolic inhibitors, such as azide, which lower intracellular ATP (Fig. 2). In contrast, significant resting whole-cell K⁺ currents are present in oocytes expressing homomeric Q52R, V59G, or R201C (homQ52R, homV59G, and homR201C, respectively) (Fig. 2 and Table 1). This result suggests that metabolism causes less block of mutant Kₐ₅P channels than wild-type channels. Whole-cell homV59G and homQ52R currents were not increased further by azide application, as if these channels are fully activated at rest and insensitive to metabolically induced changes in nucleotides. However, homR201C channels were enhanced by azide, suggesting that they are more ATP-sensitive than homV59G and homQ52R channels. For heterozygous channels (hetV59G, hetQ52R, and hetR201C), hetV59G and hetQ52R resting whole-cell currents were of similar amplitude, constituting ~65% of their hommeric values, but were far larger than wild-type currents (Fig. 2B). These resting currents were further activated by azide, reaching a final amplitude close to that of both homomeric and wild-type channels. Resting hetR201C currents were smaller than hetQ52R and hetV59G currents but still significantly larger than wild-type (Fig. 2 and Table 1). These differences in resting current probably account for the difference between the mild and severe forms of the disease because they may be expected to hyperpolarize the patients’ cells and thereby influence electrical activity and cell function to differing extents.

**The Molecular Basis of Disease Severity.** To explore the molecular basis of the different metabolic sensitivities, we examined the
ability of ATP to block wild-type and mutant channels in inside-out patches (Fig. 3). All homomeric mutant channels were substantially less sensitive to intracellular ATP than wild type, the order of potency being WT > H11022Q52R > H11022R201C > H11022V59G (Table 1). The reduced ATP sensitivity is expected to contribute to the larger resting whole-cell KATP currents. Although all homomeric mutant channels showed markedly reduced ATP sensitivity, significant differences were observed for heterozygous channels (Fig. 3B). In particular, hetQ52R and hetV59G channels were half-maximally blocked by ATP concentrations of 20–30 mM, whereas the ATP sensitivity of hetR201C channels was higher (11 mM) (Table 1). The different ATP sensitivities of the heterozygous channels parallel the variation in resting whole-cell currents observed for these channels in oocytes and underlie the difference between the mild and severe forms of disease.

In previous studies using the mouse Kir6.2 clone, we were unable to detect a significant change in the ATP sensitivity of the heterozygous channel population with the R201H mutation (4). We therefore tested the effect of the R201H mutation in the human Kir6.2 clone. We found that ATP produced a half-maximal block of homomeric R201H channels at 298 ± 25 μM (n = 5), and of the heterozygous channel population at 12.5 ± 1.1 μM (n = 5). The latter value is close to that found for hetR201C, which also causes mild disease, and significantly less than that found for mutations associated with severe disease.

In pancreatic beta cells, an increase in KATP current will hyperpolarize the plasma membrane and suppress electrical activity, which will lead to a reduction in insulin secretion and, thereby, diabetes (11). Although the ATP concentration causing half-maximal block of hetR201C channels is only slightly greater than that found for wild-type channels (11 μM vs. 7 μM) (Table 1), the magnitude of the relative current at 0.1 mM ATP is ~2.5-fold larger (0.1 ± 0.02, n = 5, compared with 0.04 ± 0.01, n = 6, respectively) (Fig. 3B). Because [ATP] is unlikely to fall below 0.1 mM in beta cells, even when extracellular glucose is low (12), the difference in wild-type and mutant KATP current at this (and higher) ATP concentration probably explains why the R201C and R201H mutations result in neonatal diabetes.
Table 1. Macroscopic and single-channel properties of PNDM mutations

<table>
<thead>
<tr>
<th>Mutations</th>
<th>Irest, µA (n)</th>
<th>IC50 ATP, µM (n)</th>
<th>PO(0) (n)</th>
<th>i, pA (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.07 ± 0.01 (8)</td>
<td>7.0 ± 1.1 (6)</td>
<td>0.53 ± 0.02 (8)</td>
<td>4.1 ± 0.2 (3)</td>
</tr>
<tr>
<td>homQ52R</td>
<td>5.49 ± 0.47 (10)*</td>
<td>84 ± 12 (6)*</td>
<td>0.83 ± 0.01 (8)*</td>
<td>4.2 ± 0.2 (6)*</td>
</tr>
<tr>
<td>homV59G</td>
<td>6.42 ± 0.24 (10)*</td>
<td>7,400 ± 1,500 (6)*</td>
<td>0.83 ± 0.01 (8)*</td>
<td>4.0 ± 0.1 (6)*</td>
</tr>
<tr>
<td>homR201C</td>
<td>3.73 ± 0.12 (12)*</td>
<td>106 ± 12 (6)*</td>
<td>0.60 ± 0.03 (9)*</td>
<td>3.9 ± 0.3 (6)*</td>
</tr>
<tr>
<td>hetQ52R</td>
<td>3.66 ± 0.39 (9)*</td>
<td>23 ± 3 (6)*</td>
<td>0.70 ± 0.03 (9)*</td>
<td>ND</td>
</tr>
<tr>
<td>hetV59G</td>
<td>4.19 ± 0.32 (9)*</td>
<td>26 ± 6 (6)*</td>
<td>0.70 ± 0.03 (9)*</td>
<td>ND</td>
</tr>
<tr>
<td>hetR201C</td>
<td>1.22 ± 0.13 (12)*</td>
<td>11 ± 2 (5)*</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Mean values of resting whole-cell KATP current (Irest), ATP concentration producing half-maximal block the channel (IC50), intrinsic open probability (i.e., in the absence of ATP) PO(0), and single channel current at ~60 mV (i). Statistical significance against wild-type is indicated: *, P < 0.001; †, P < 0.01; §, P < 0.05; †, not significant. ND, not determined.

Effects of PNDM Mutations on Single-Channel Kinetics. Why does the Q52R mutation cause a greater shift in the ATP sensitivity of the heterozygous channel than the R201C mutation, despite the similar ATP sensitivities of the homomeric channels? To answer this question, we recorded single-channel currents in inside-out membrane patches in ATP-free solution, where the intrinsic gating of the channel can be assessed. Fig. 4 shows that the open probabilities of the mutant channels in the unliganded state, PO(0), were strikingly different. Thus, the PO(0) of homomeric channels containing the R201C mutation was not significantly different from wild-type, whereas that of homomeric channels containing the R201C mutation was substantially greater (Table 1). It is well documented that an increase in PO(0) reduces the ability of ATP to close the KATP channel (30, 31). Thus, mutations at residue 201, which lies within the putative ATP-binding site (8), probably act by reducing ATP binding per se, whereas the Q52R and V59G mutations appear to decrease ATP sensitivity indirectly, by favoring the open conformation of the channel. Because V59G channels have a much lower ATP sensitivity than Q52R channels, despite a similar PO(0), the V59G mutation is also likely to affect ATP binding and/or the mechanism by which ATP binding is transduced into channel closure. The data are also consistent with a role for the slide helix, within which residue 59 resides, in Kir channel gating.

Lipids, such as PIP2, increase the PO(0) and reduce the ATP sensitivity of the KATP channel (32). To determine whether the V59G mutation affects PO(0) directly or indirectly by means of an increase in PIP2 binding, we used neomycin, a polycation that binds to PIP2, closes KATP channels and has been used to evaluate PIP2 affinity (33). In 3/3 patches, 100 µM neomycin did not alter Kir6.2-V59G/SUR1 currents over 5-10 min (data not shown). This finding is consistent with the idea that the V59G mutation affects PO(0) directly, rendering gating insensitive to PIP2 modulation.

Given that the KATP channel pore is composed of four Kir6.2 subunits (2), in the heterozygous state most channels will contain a mixture of wild-type and mutant subunits (Fig. 1C). Because ATP binding to a single subunit is sufficient to close the channel with reduced ATP sensitivity, which also have a normal ECG (29).
Kir6.2-V59G/H20862

ified Hill equation containing 1/H20862

n (red filled circles, [ATP] and KATP conductance, membrane face as indicated. (Line indicates the zero current level. ATP was applied to the intracellular IC50 the absence of nucleotide, for wild type (open blue circles) and hetR201C (filled red

gons) channels.

circles), hetQ52R (filled black squares), and hetV59G (filled green hexa-

ons) channels.

Fig. 3. Effects of mutations on KATP channel ATP sensitivity. (A) KATP current recorded in response a succession of voltage ramps from −110 to +100 mV to an inside-out patch excised from a Xenopus oocyte coexpressing SUR1 and either wild-type or mutant Kir6.2, as indicated. The dotted line indicates the zero current level. ATP was applied to the intracellular membrane face as indicated. (B) (Upper Left) Mean relationship between [ATP] and KATP conductance, G, expressed relative to the conductance in the absence of nucleotide, G0, for Kir6.2/SUR1 (open blue circles, n = 6), and heterozygous (filled red circles, n = 6) and homomeric (filled black squares, n = 6) Kir6.2-R201C/SUR1 channels. The smooth curves are the best fit to the Hill equation. For wild-type, IC50 = 6.6 μM and h = 1.1. For heterozygous R201C, IC50 = 10.4 μM and h = 1.0. For homoromic R201C, IC50 = 102 μM and h = 1.3. (Upper Right) Mean relationship between [ATP] and KATP conductance expressed relative to the conductance in the absence of nucleotide for Kir6.2/SUR1 (open blue circles, n = 6) and heterozygous (red filled circles, n = 6) and homomer (black filled squares, n = 6) Kir6.2-V59G/SUR1 channels. The smooth curves are the best fit to a modified Hill equation containing 1/16 of homomeric channels. For wild-type, IC50 = 6.6 μM and h = 1.1. For heterozygous V59G, IC50 = 26 μM and h = 1.18. For homoromic V59G, IC50 = 8.1 mM and h = 0.75. (Lower Left) Mean relationship between [ATP] and KATP conductance expressed relative to the conductance in the absence of nucleotide for Kir6.2/SUR1 (open blue circles, n = 6) and heterozygous (filled red circles, n = 6) and homomer (black filled squares, n = 6) Kir6.2-Q52R/SUR1 channels. The smooth curves are the best fit to the Hill equation. For wild-type, IC50 = 6.6 μM and h = 1.1. For heterozygous Q52, IC50 = 21 μM and h = 1.2. For homoromic Q52R, IC50 = 83 μM and h = 1.7. (Lower Right) Mean relationship between [ATP] and KATP conductance expressed relative to the conductance in the absence of nucleotide for wild type (open blue circles) and hetR201C (filled red circles), hetQ52R (filled black squares), and hetV59G (filled green hexagons) channels.

Fig. 4. Effects of mutations on single-channel currents. Single KATP channel currents were recorded at −60 mV from inside-out patches excised from oocytes coinjected with mRNAs encoding SUR1 plus either wild-type or mutant Kir6.2, as indicated.

Mutations May Differentially Alter Sulfonylurea Efficacy. Our finding that Kir6.2 mutations produce neonatal diabetes by different molecular mechanisms may have implications for therapy. We observed that 500 μM tolbutamide blocked azide-activated whole-cell currents by 89 ± 1% (n = 12), 65 ± 5% (n = 9), and 100 mV from inside-out patches excised from oocytes coinjected with mRNAs encoding SUR1 plus either wild-type or mutant Kir6.2, as indicated.

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Proks et al.
41 ± 2% (n = 9) for hetR201C, hetQ52R, and hetV59G channels, respectively. This difference is consistent with previous reports that mutations that enhance intrinsic K\textsubscript{ATP} channel opening reduce the inhibitory efficacy of sulfonylureas (30). Thus, our results indicate that, whereas sulfonylureas may produce a greater reduction in ATP-sensitivity of heterozygous K\textsubscript{ATP} channels result in neonatal diabetes alone, whereas those that produce a greater reduction in ATP-sensitivity are associated with additional symptoms. The molecular mechanism by which a mutation affects the channel ATP sensitivity determines the severity of its effect in the heterozygous state, with those mutations that influence gating producing larger effects on ATP sensitivity, and a more severe disease phenotype, than those that lie in the putative ATP-binding site. Our results also suggest that the efficacy of sulfonylurea therapy may vary with genotype.

Conclusions

Our results demonstrate that KCNJ11 mutations that cause a small decrease in the ATP sensitivity of heterozygous K\textsubscript{ATP} channels may provide a valuable alternative to insulin injections for patients with mild mutations (like R201) (4, 6), those patients with mutations affecting intrinsic gating may require more intensive drug therapy or a combination of drug and insulin therapy.

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