A gating mutation at the internal mouth of the Kir6.2 pore is associated with DEND syndrome

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Inwardly rectifying potassium (Kir) channels control cell membrane K⁺ fluxes and electrical signalling 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. However, the I296L mutation also results in developmental delay, muscle weakness and epilepsy. We investigated the functional effects of the I296L mutation by expressing wild-type or mutant Kir6.2/SUR1 channels in *Xenopus* oocytes. The mutation caused a marked increase in resting whole-cell K_{ATP} currents by reducing channel inhibition by ATP, in both homomeric and simulated heterozygous states. Kinetic analysis showed that the mutation impaired ATP sensitivity indirectly, by stabilizing the open state of the channel and possibly also by means of an allosteric effect on ATP binding and/or transduction. The results implicate a new region in Kir-channel gating and suggest that disease severity is correlated with the extent of reduction in ATP sensitivity.

Keywords: neonatal diabetes; ATP-sensitive K⁺ channel; sulphonylurea; Kir6.2; gating; ATP block

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INTRODUCTION

ATP-sensitive potassium (K_{ATP}) channels control electrical signalling in diverse cell types by coupling cellular metabolism to potassium movement across cell membranes (Seino & Miki, 2003). In pancreatic β -cells, they link changes in blood glucose

concentration to insulin secretion. In the brain, they contribute to glucose sensing and seizure protection; in cardiac muscle, they protect against ischaemic stress; in skeletal muscle, they influence glucose uptake; and in vascular smooth muscle, they regulate muscle tone. K_{ATP} channels comprise four pore-forming Kir6.2 subunits and four regulatory sulphonylurea receptor (SUR) subunits (Ashcroft & Gribble, 1998). ATP binding to Kir6.2 closes the channel, whereas the interaction of Mg-nucleotides with SUR opens it (Nichols *et al*, 1996; Tucker *et al*, 1997). Consequently, increased metabolism leads to channel closure, membrane depolarization and electrical activity; in contrast, metabolic inhibition opens K_{ATP} channels and suppresses electrical activity (Ashcroft *et al*, 1984).

Recently, it was found that heterozygous mutations in Kir6.2 (*KCNJ11*) cause permanent neonatal diabetes (PNDM) alone or neonatal diabetes in association with developmental delay, epilepsy or muscle weakness (DEND syndrome; Gloyn *et al*, 2004; Sagen *et al*, 2004). Functional analysis of mutations at R201, which cause PNDM, showed that they increased whole-cell K_{ATP} currents by reducing channel inhibition by ATP (Gloyn *et al*, 2004; Proks *et al*, 2004). As this residue lies in the putative ATP-binding site, it may mediate its effect by impairing ATP binding.

A severe phenotype was associated with an isoleucine to leucine substitution at residue 296 of Kir6.2. The patient with this mutation had muscle weakness, generalized complex epilepsy, and motor and mental developmental delay that were so severe that she could not walk or speak at the age of 17 years (Gloyn *et al*, 2004). Residue I296 lies some distance in the primary sequence from residues that form the putative ATP-binding site (Fig 1) and mutations at this position may therefore act by a different mechanism to R201H. Functional analysis of cloned channels confirmed this idea and showed that I296L impairs ATP sensitivity indirectly, by biasing the channel conformation towards the open state. Thus, our results implicate a new region of Kir channels in gating. They also support the idea that Kir6.2 mutations that produce a marked decrease in ATP inhibition in the pseudo-heterozygous state give rise to DEND syndrome, whereas those that cause a less severe reduction produce PNDM alone (Proks *et al*, 2004).

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Fig 1 | Location of I296 in Kir6.2. (A) Molecular model of Kir6.2, viewed from the side. For clarity, each subunit is shown in a different colour and only two transmembrane domains (TMs) and two cytosolic domains are illustrated. I296 is shown in a ball-and-stick format and ATP in yellow. (B) Molecular model of the Kir6.2 tetramer, viewed from below. For clarity, the TMs have been removed, and the N- and C-terminal domains are shown in a ribbon and backbone format, respectively. ATP (yellow) is shown docked into its four binding sites. (C) Expanded views of the orientation of residue 296, with either isoleucine (blue) or leucine (yellow) at this position. Left, viewed from the side; right, viewed from above.

RESULTS AND DISCUSSION

Location of I296

Fig 1 illustrates the position of I296 in a structural model of Kir6.2 (Antcliff *et al*, 2005) based on the crystal structures of the bacterial homologue KirBac1.1 (Kuo *et al*, 2003) and the cytosolic domains of the related eukaryotic channel Kir3.1 (Nishida & MacKinnon, 2002). In the three-dimensional structure, I296 lies distant from the putative ATP-binding site. It is located ~ 7 Å below the plane of the membrane, at the internal mouth of the transmembrane pore, and forms a hydrophobic ring with a diameter of ~ 11 Å that defines the narrowest part of the cytosolic pore.

Effects on whole-cell currents

When expressed in *Xenopus* oocytes, wild-type Kir6.2/SUR1 channels are normally closed because of the high intracellular ATP concentration ($[ATP]_i$), and open only in response to metabolic inhibitors, such as azide, which lower $[ATP]_i$ (Fig 2; Gribble *et al*, 1997). In contrast, significant resting K⁺ currents were present in oocytes expressing homomeric (*hom*) I296L channels (Fig 2). These currents were not increased by azide, suggesting that the channel is fully open at resting $[ATP]_i$ in the oocyte. They were also insensitive to tolbutamide, which blocks

wild-type K_{ATP} channels. Co-injection of mutant and wild-type Kir6.2, to simulate the heterozygous state, produced resting currents (*het*I296L) that were much greater than wild-type, and were further increased by azide and only partially blocked by tolbutamide (Fig 2, Table 1).

The reduced sensitivity of *het*I296L current to 0.5 mM tolbutamide, a concentration that causes maximal block of wild-type channels, suggests that it may not be possible to treat diabetes in patients carrying this mutation with oral sulphonylureas alone, as has been reported for mutations that cause PNDM (Sagen *et al*, 2004). However, the ability of tolbutamide to block 50% of the current suggests that sulphonylureas may still be able to alleviate some of the symptoms of DEND syndrome that involve peripheral nerve and muscle. A combination therapy of insulin and sulphonylureas may therefore be beneficial.

Effects on K_{ATP}-channel ATP sensitivity

The increase in resting whole-cell K_{ATP} currents caused by the I296L mutation suggests that it may reduce the channel ATP sensitivity. To explore this idea, we first measured the ATP concentration–response curves in the absence of Mg²⁺, to avoid the complication caused by the stimulatory effects of

Mg-nucleotides. Fig 3A shows that homI296L and hetI296L channels are blocked half-maximally by 3.4 mM and 57 μ M ATP, respectively, compared with 7 μ M ATP for wild-type channels (Table 1). It is well established that, even in the absence of Mg^{2+} , SUR1 enhances the ATP sensitivity of the K_{ATP} channel (Tucker *et al*, 1997; Gribble *et al*, 1998). However, as Fig 3B shows, the ATP sensitivity of Kir6.2 Δ C, expressed in the absence of SUR1, was also impaired by the I296L mutation (Table 2). This suggests that the reduction in ATP inhibition of I296L channels (in Mg^{2+} -free solution) is intrinsic to Kir6.2 rather than a result of impaired regulation by SUR1.

In the intact pancreatic β -cell, the effect of the I296L mutation on K_{ATP} -channel ATP sensitivity may be modified by the stimulatory effect of MgATP, which is mediated by the nucleotide-binding domains of SUR1 (Nichols *et al*, 1996; Gribble *et al*, 1997, 1998). Thus, we next compared the ATP concentration–inhibition curves for wild-type, hetI296L and homI296L channels in the presence of Mg^{2+} (Fig 4A, Table 1). In the presence of Mg^{2+} , homI296L currents were blocked by less than 10%, even at ATP

concentrations as high as 10 mM. Although there was no substantial effect of Mg^{2+} on the IC_{50} for the ATP block of hetI296L (64 μ M and 57 μ M in the presence and absence of Mg^{2+} , respectively; Table 1), the fraction of the unblocked current at high ATP concentrations was much greater in the presence of the divalent cation, being 36% versus 7% at 1 mM ATP (Table 1). As Mg^{2+} is always present inside the cell, and ATP concentrations are probably 1 mM or above (Gribble *et al*, 2000), this suggests that there will be a substantial increase in the resting whole-cell K_{ATP} currents for both hetI296L and homI296L channels, as observed experimentally (Fig 2).

Mechanism of reduction in ATP sensitivity

Residues that reduce the sensitivity of the K_{ATP} channel to ATP include those that impair ATP binding, and those that influence the intrinsic (unliganded) gating of the channel (Trapp *et al*, 1998; Enkvetchakul *et al*, 2001). Because I296 lies distant from the putative ATP-binding site (Fig 1), we examined the effect of the I296L mutation on single-channel currents in the absence of ATP, where intrinsic gating can be assessed. The intrinsic open probability (P_o) was markedly increased for both homI296L and hetI296L channels (Fig 4B, Table 1), which suggests that the mutation enhances the open-state stability of the channel. This effect will contribute to the loss of ATP sensitivity. A similar molecular mechanism underlies the reduced ATP sensitivity conferred by the Q52R and V59G mutations associated with DEND syndrome (Proks *et al*, 2004). The marked reduction in the tolbutamide block of whole-cell currents is probably secondary to the increase in intrinsic open probability (Trapp *et al*, 1998).

Lipids such as phosphatidylinositol 4,5-bisphosphate (PIP_2) increase P_o and reduce the ATP sensitivity of the K_{ATP} channel (Shyng & Nichols, 1998; Fan & Makielski, 1999). To determine whether the I296L mutation affects gating directly or indirectly by an increase in PIP_2 binding, we used neomycin, a polycation that binds to PIP_2 , closes the wild-type K_{ATP} channel and has been used to measure its PIP_2 affinity (Krauter *et al*, 2001). In five out of five patches, 100 μ M neomycin did not alter homI296L currents for more than 5–10 min (see the supplementary information online). This lack of effect is consistent with the idea that the I296L mutation affects intrinsic gating directly, rendering it insensitive to PIP_2 modulation.

Is the increase in P_o able to account fully for the reduced ATP sensitivity of I296L channels? To further explore this question, we turned to a kinetic model (see the supplementary information online). This model assumes independent mixing of wild-type and mutant Kir6.2 subunits within the tetrameric channel (Shyng & Nichols, 1997), that ATP binding to one subunit can close the channel (Markworth *et al*, 2000) and that interactions between subunits during gating do not occur (Enkvetchakul *et al*, 2001). There was a reasonably good correlation between the measured and predicted curves for hetKir6.2 Δ C-I296L channels (supplementary Fig S2 online): the IC_{50} values are 805 and 880 μ M, respectively. Thus, in this case, the effect of the I296L mutation on ATP inhibition can be explained entirely as a secondary consequence of the altered channel kinetics. The predicted ATP concentration–inhibition curve for hetKir6.2-I296L/SUR1 channels had an IC_{50} (20 μ M) that was considerably less than that measured experimentally (57 μ M). There may be several reasons for this difference. It may reflect interactions between subunits (which are

Fig 2 | Comparison of whole-cell currents of wild-type and mutant K_{ATP} channels. (A) Whole-cell currents recorded from *Xenopus* oocytes coexpressing SUR1 and either Kir6.2 or Kir6.2-I296L (homI296L), or both (hetI296L), in response to voltage steps of ± 20 mV from a holding potential of -10 mV. Arrows indicate the start of azide application. (B) Mean steady-state whole-cell currents evoked by a voltage step from -10 to -30 mV before (white bars) and after application of 3 mM azide (black bars) and in the presence of 3 mM azide and 0.5 mM tolbutamide (tolb; grey bars). The number of oocytes is given above the bars.

Table 1 | Mean data for wild-type and mutant Kir6.2/SUR1 channels

Mutation	I_{rest}	IC_{50} (Mg-free)	h (Mg-free)	IC_{50} (2 mM Mg)	h (2 mM Mg)	P_o
Kir6.2	$0.14 \pm 0.03 \mu A$ ($n = 8$)	$7.0 \pm 1.1 \mu M$ ($n = 6$)	1.11 ± 0.11 ($n = 6$)	$13.0 \pm 0.7 \mu M$ ($n = 6$)	1.02 ± 0.05 ($n = 6$)	0.53 ± 0.02 ($n = 8$)
Kir6.2-I296L (hetero)	$2.72 \pm 0.34 \mu A$ ($n = 9$)***	$57 \pm 7 \mu M$ ($n = 5$)***	1.00 ± 0.06 ($n = 5$)	$64 \pm 12 \mu M$ ($n = 9$)**	0.86 ± 0.12 ($n = 9$)	0.72 ± 0.02 ($n = 9$)***
Kir6.2-I296L (homo)	$3.79 \pm 0.37 \mu A$ ($n = 9$)***	$3.4 \pm 0.9 mM$ ($n = 5$)***	0.69 ± 0.06 ($n = 5$)	NM***	NM***	0.85 ± 0.01 ($n = 7$)***

Mean values of resting whole-cell K_{ATP} current (I_{rest}), ATP concentration producing half-maximal of the channel (IC_{50}), Hill coefficient (h) and intrinsic open probability in the absence of ATP (P_o) (for heterozygous channels this refers to the average intrinsic open probability) are provided. Homo, homomeric channels; hetero, heterozygous channels; NM, not measurable (as too ATP insensitive). The number of patches is indicated in parentheses. Statistical significance against wild-type is indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

not included in the model). Alternatively, in addition to affecting gating, the mutation might influence ATP binding and/or the mechanism by which binding is transduced into ATP inhibition.

In our structural model, I296 lies in a region of the channel not previously implicated in gating, which illustrates the value of naturally occurring mutations in understanding channel function. However, I296 lies within a cytosolic loop that interacts with two regions of the channel that are involved in gating: the ‘slide helix’ and the region of the carboxy-terminal domain that connects to the outer transmembrane helix (Antcliff *et al*, 2005). It is possible that the mutation of I296 influences open-state stability of the channel by affecting these interactions. Indeed, the crystal structures of Kir channels suggest that these loops are flexible and may move during gating: residues in the equivalent structural position to I296L form a narrow opening of 5.7 Å (Kir2.1) and 9.0 Å (Kir3.1) diameter (Pegan *et al*, 2005).

Functional implications

The large amplitude of hetI296L currents in the presence of physiological levels of MgATP is expected to cause hyperpolarization of native cells, thereby reducing their electrical activity. In pancreatic β -cells, this will lead to a reduction in

◀ **Fig 3** | Comparison of the ATP sensitivity of wild-type and mutant K_{ATP} channels in excised patches. (A) (Top) K_{ATP} current recorded in response to successive voltage ramps from -110 to +100 mV in an inside-out patch excised from a *Xenopus* oocyte coexpressing SUR1 and either Kir6.2 or Kir6.2-I296L. The dotted line indicates the zero current level. The bar indicates ATP application. (Bottom) Mean relationship between [ATP] and K_{ATP} conductance (G) expressed relative to the conductance in the absence of nucleotide (G_c) for Kir6.2/SUR1 (blue filled circle, $n = 6$) and for heterozygous (red filled circle, $n = 5$) or homomeric (filled square, $n = 5$) Kir6.2-I296L/SUR1 channels. The smooth curves are the best fit to equation (1), using the parameters given in supplementary Table 1 online. (B) (Top) Kir6.2 Δ C or Kir6.2 Δ C-I296L currents recorded in response to voltage ramps from -110 to +100 mV in an inside-out patch. (Bottom) Mean relationship between [ATP] and K_{ATP} conductance (G) expressed relative to the conductance in the absence of nucleotide (G_c) for Kir6.2 Δ C channels (blue filled circle, $n = 6$) and for heterozygous (red filled circle, $n = 5$) or homomeric Kir6.2 Δ C-I296L channels (filled square, $n = 5$). The smooth curves are the best fit to equation (1), using the parameters given in supplementary Fig S1 online.

Table 2|Mean data for wild-type and mutant Kir6.2ΔC channels expressed in the absence of SUR1

Mutation	IC ₅₀	h	P _o
Kir6.2ΔC	197 ± 16 μM (n = 6)	0.97 ± 0.02 (n = 6)	0.08 ± 0.02 (n = 6)
Kir6.2ΔC-I296L (hetero)	805 ± 137 μM (n = 5)***	1.18 ± 0.05 (n = 5)	NM
Kir6.2ΔC-I296L (homo)	28 ± 14 mM (n = 5)***	0.98 ± 0.19 (n = 5)	0.70 ± 0.02 (n = 7)***

Mean values of IC₅₀, Hill coefficient (h) and intrinsic open probability (P_o) are provided. NM, not measurable. *P < 0.05, **P < 0.01 and ***P < 0.001.

Fig 4|(A) ATP concentration–response relations in the presence of Mg^{2+} . Mean relationship between $[MgATP]$ and K_{ATP} conductance (G) expressed relative to the conductance in the absence of nucleotide (G_c) for Kir6.2/SUR1 (blue filled circle, $n = 6$) and heterozygous (red filled circle, $n = 5$) or homomeric (filled square, $n = 5$) Kir6.2-I296L/SUR1 channels. The smooth curves through the wild-type and hetI296L data represent the best fit to equation (1), using the parameters given in supplementary Table 1 online. For hetI296L data, the fitted value of a was 0.28. The curve through the homI296L data was drawn by hand. (B) Kinetics of wild-type and mutant K_{ATP} channels. Representative single K_{ATP} -channel currents recorded at -60 mV from inside-out patches from oocytes expressing SUR1 and either wild-type or mutant Kir6.2.

insulin secretion and thus to diabetes (Ashcroft & Rorsman, 2004). In many muscle and nerve tissues, K_{ATP} channels are normally shut and open only under metabolic stress (Seino & Miki, 2003). Thus, a greater reduction in ATP sensitivity is required to increase the resting K_{ATP} current enough to influence electrical activity. This may explain why the mutations at residue R201, which produce only a small decrease in the ATP sensitivity of heterozygous channels, result in neonatal diabetes alone (Gloyn et al, 2004; Proks et al, 2004), whereas the I296L mutation, which

causes a much greater decrease in ATP sensitivity, has a more severe phenotype that involves also muscle and the central nervous system. In support of this idea, mutations at Q52 and V59 that cause large reductions in ATP sensitivity also give rise to DEND syndrome (Proks et al, 2004).

METHODS

Functional studies. Human Kir6.2 (GenBank NM000525; E23 and I377) and rat SUR1 (GenBank L40624) were used in this study. Wild-type or mutant Kir6.2, and Kir6.2 lacking the final 36 residues (Kir6.2ΔC; Tucker et al, 1997), were coexpressed with SUR1 in *Xenopus laevis* oocytes (see the supplementary information online). Whole-cell currents were recorded using the two-electrode voltage-clamp method (Gribble et al, 1997) in a solution containing (in mM) 90 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES (pH 7.4 with KOH) and 3 mM sodium azide, as indicated. Macroscopic currents were recorded from giant, excised inside-out patches (Gribble et al, 1997). The pipette solution contained (in mM) 140 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$ and 10 HEPES (pH 7.4 with KOH). The Mg -free internal (bath) solution contained (in mM) 107 KCl, 1 K_2SO_4 , 10 EGTA, 10 HEPES (pH 7.2 with KOH) and K_2ATP , as indicated. Mg -containing solution was made by adding 2 mM $MgCl_2$ to the internal solution and using $MgATP$ rather than ATP .

The macroscopic slope conductance was measured between -20 and -100 mV (Gribble et al, 1997). The ATP concentration–response curves were fit with the Hill equation (equation 1):

$$G/G_c = a + (1 - a)/(1 + ([ATP]/IC_{50})^h) \quad (1)$$

where $[ATP]$ is the ATP concentration, IC_{50} is the ATP concentration at which inhibition is half-maximal, h is the slope factor (Hill coefficient) and a represents the fraction of unblocked current at saturating $[ATP]$. In all experiments, except for hetI26L channels studied in the presence of Mg^{2+} , a was taken as zero. To control for possible rundown, G_c was taken as the mean of the conductance in control solution before and after ATP application.

Single-channel currents were recorded at -60 mV from small inside-out patches, as described previously (Trapp et al, 1998). Open probability was determined from current records of ~ 1 min duration as I/iN , where I is the macroscopic current, i is the single-channel current amplitude and N is the number of channels. For heterozygous channels, the mean P_o ($\langle P_o \rangle$) represents the average of all types of channel. Data are mean $\pm$ s.e.m.

Molecular modelling. All sequence alignments were carried out using Clustal X (Jeanmougin et al, 1998). Models of mouse Kir6.2 (residues 32–358) were generated as described previously (Antcliff et al, 2005), using Modeller v6.2 (Sali & Blundell, 1993). The mutant models were generated using InsightII (www.accelrys.com), by replacing isoleucine 296 with leucine. The mutant residue was superpositioned over the wild type, thereby maintaining the

spatial orientation within the model. The quality and stereochemical properties of the original and mutant models were evaluated using ProCheck (Laskowski *et al*, 1993). ATP was docked in the C-terminal domain using the protocol described previously (Trapp *et al*, 2003). Docking was performed using AutoDock 3.0.5 (Goodsell *et al*, 1996). Structural diagrams were generated using VMD (Humphrey *et al*, 1996).

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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