A universally conserved residue in the SUR1 subunit of the \( \text{K}_{\text{ATP}} \) channel is essential for translating nucleotide binding at SUR1 into channel opening

Heidi de Wet\(^1\), Kenju Shimomura\(^1\), Jussi Aittoniemi\(^1\,2\), Nawaz Ahmad\(^1\), Mathilde Lafond\(^1\), Mark S.P. Sansom\(^2\) and Frances M. Ashcroft\(^1\)

\(^1\)Henry Wellcome Centre for Gene Function, Department of Physiology, Anatomy and Genetics, University of Oxford, Parks Road, Oxford OX1 3PT, UK
\(^2\)Department of Biochemistry, South Parks Road, University of Oxford, Parks Road, Oxford OX1 3QU, UK

**Key points**

- The sulphonylurea receptor (SUR1) subunit of the ATP-sensitive potassium (\( \text{K}_{\text{ATP}} \)) channel is a member of the ATP-binding cassette (ABC) protein family. Binding of MgADP to nucleotide-binding domain 2 (NBD2) is critical for channel activation.
- We identified a residue in the SUR1 subunit of the \( \text{K}_{\text{ATP}} \) channel that is essential for translating nucleotide binding to SUR1 into activation of the channel pore.
- The ability of ATP to block \( \text{K}_{\text{ATP}} \) channel activity by binding to the Kir6.2 subunit of the channel was also altered by the mutation. This effect was dependent on the integrity of the NBDs of SUR1. This suggests SUR1 also modulates nucleotide inhibition of the channel at Kir6.2.
- G1401 in SUR1 is one of 23 residues which are conserved throughout all ABC transporter proteins suggesting that it may have a universal role in coupling substrate binding to protein function.

**Abstract** The sulphonylurea receptor (SUR1) subunit of the ATP-sensitive potassium (\( \text{K}_{\text{ATP}} \)) channel is a member of the ATP-binding cassette (ABC) protein family. Binding of MgADP to nucleotide-binding domain 2 (NBD2) is critical for channel activation. We identified a residue in NBD2 (G1401) that is fully conserved among ABC proteins and whose functional importance is unknown. Homology modelling places G1401 on the outer surface of the protein, distant from the nucleotide-binding site. The ATPase activity of purified SUR1-NBD2-G1410R (bound to maltose-binding protein) was slightly inhibited when compared to the wild-type protein, but its inhibition by MgADP was unchanged, indicating that MgADP binding is not altered. However, MgADP activation of channel activity was abolished. This implies that the G1401R mutation impairs the mechanism by which MgADP binding to NBD2 is translated into opening of the \( \text{K}_{\text{ATP}} \) channel pore. The location of G1401 would be consistent with interaction of this residue with the pore-forming Kir6.2 subunit. Channel activity in the presence of MgATP reflects the balance between the stimulatory (at SUR1) and inhibitory (at Kir6.2) effects of nucleotides. Mutant channels were 2.5-fold less sensitive to MgATP inhibition and not activated by MgATP. This suggests that ATP block of the channel is reduced by the SUR1 mutation. Interestingly, this

H. de Wet and K. Shimomura contributed equally to the work.

Introduction

ATP-binding cassette (ABC) proteins constitute the largest known family of transmembrane proteins (Dean et al. 2001). They serve as ATP-dependent transporters, channels and channel regulators in both prokaryotes and eukaryotes. Many human ABC protein genes play a causative role in disease, including cystic fibrosis (ABCC7), congenital hyperinsulaeemia (ABCC8), neonatal diabetes (ABCC8), retinal degeneration (ABCA4), bile transport disorders (ABCB4), anaemia (ABCB1) and adrenoleukodystrophy (ABCD1). Other ABC proteins are involved in drug resistance (e.g. ABCB1) and are implicated in disease, including congenital hyperinsulinaemia (HI), hyperinsulinaemia of infancy; K\textsubscript{ATP} channel, ATP-sensitive potassium channel; NBD, nucleotide binding domain; NBS, nucleotide binding site; ND, neonatal diabetes; SUR, sulphonylurea receptor.

Effect was dependent on the functional integrity of the NBDs. These results therefore suggest that SUR1 modulates both nucleotide inhibition and activation of the K\textsubscript{ATP} channel.

(Received 4 May 2012; accepted after revision 13 July 2012; first published online 16 July 2012)

Corresponding author F. Ashcroft: Department of Physiology, anatomy and Genetics, Parks Road, Oxford OX1 3PT, UK. Email: frances.ashcroft@dpag.ox.ac.uk

Abbreviations ABC, ATP-binding cassette; HI, hyperinsulinaeemia of infancy; K\textsubscript{ATP}, channel, ATP-sensitive potassium channel; NBD, nucleotide binding domain; NBS, nucleotide binding site; ND, neonatal diabetes; SUR, sulphonylurea receptor.

The sulphonylurea receptor (SUR) is a member of the ABCC subfamily that – uniquely among ABC proteins – functions as an ion channel regulator. It also has an extra domain (TMD0), consisting of five transmembrane helices, at its N-terminus. Four SUR subunits associate with four Kir6.x subunits (Kir6.2 or Kir6.1) to form a large hetero-octameric complex, the ATP-sensitive potassium (K\textsubscript{ATP}) channel (Nichols, 2006). These channels are so-called because they are inhibited by ATP and activated by Mg-nucleotides (MgATP, MgADP). Kir6.x belongs to the inwardly rectifying potassium channel family and forms the channel pore. ATP binding to this subunit closes the channel (Tucker et al. 1997). SUR endows the channel with sensitivity to the stimulatory effects of Mg-nucleotides, as well as to activation by K\textsubscript{ATP} channel openers, and inhibition by sulphonylurea drugs. There are two isoforms, SUR1 and SUR2 (Aguilar-Bryan et al. 1995; Inagaki et al. 1996; Isomoto et al. 1996) which confer different nucleotide and drug sensitivities.

In pancreatic \(\beta\)-cells, K\textsubscript{ATP} channels link cellular metabolism to insulin release by sensing changes in adenine nucleotide concentrations. At low blood glucose (and cellular ATP), the channel is open and K\textsuperscript{+} efflux through the open pore keeps the membrane hyperpolarized, preventing electrical activity and insulin secretion. When plasma glucose rises, increased metabolic generation of ATP by the \(\beta\)-cell leads to closure of K\textsubscript{ATP} channels, which initiates electrical activity, calcium influx and, thereby, insulin secretion. Loss-of-function mutations in either of the two subunits of the channel, Kir6.2 (\(\text{KCNJ}11\)) or SUR1 (\(\text{ABCC}8\)), cause persistent membrane depolarisation and hyperinsulinaemia of infancy (HI); conversely, gain-of-function mutations lead to impaired insulin secretion and diabetes that presents within the first 6 months of life (neonatal diabetes, ND) (Hattersley & Ashcroft, 2005; Ashcroft, 2007). Some of these mutations act by enhancing (ND: Proks et al. 2005; Babenko et al. 2006) or inhibiting (HI: Nichols et al. 1996; Shyng et al., 1997) Mg-nucleotide activation. Mutations at residue E1507, which is implicated in ATP hydrolysis, can even cause either neonatal diabetes or hyperinsulinaemia, depending on the mutation (Mannikko et al. 2011).

A key question is how nucleotide interaction with the NBSs of SUR1 regulates channel activity. It is hypothesised that the presence of MgADP at NBS2 stimulates channel opening and that MgATP must first be hydrolysed to MgADP before channel activity is stimulated (Zingman et al. 2001). The two NBSs are also thought to work cooperatively, with MgADP binding to NBS1 enhancing ATP binding to NBS2 (Ueda et al. 1997). However, how the presence of MgADP at NBS2 is transduced into opening of the Kir6.2 pore is unclear. Given the overall structural similarity of the NBSs of ABC proteins, it seems likely that...
nucleotide binding/hydrolysis induces a conformational change in all ABC proteins that is usually coupled to substrate transport but that in SUR has been co-opted to cause channel regulation. This suggests that residues invariant across all ABC proteins might be involved in this process, as is the case for nucleotide binding and hydrolysis. In this paper, we identify a residue common to all ABC proteins that impairs Mg-nucleotide activation of the K<sub>ATP</sub> channel when mutated. It seems likely that this mutation influences the mechanism by which the presence of MgADP at NBD2 of SUR1 is translated into channel opening.

Methods

Sequence analysis

We studied the conservation of individual SUR1 residues among 12 sequences of the ABCB subfamily (SUR, MRP, CFTR) and selected other eukaryotic (ABCA1, Pgp, Tap1, Tap2) and prokaryotic (Sav1866, MsbA, BtuCD, ModBC, Hi1470/71) ABC transporters. Human SUR1 was aligned with the sequences of each class using ClustalX2 (Larkin et al. 2007) without manual curation. We used the Gonnet Protein Weight Matrix with default parameters. Residue-wise conservation of SUR1 within the different classes (ABCC, eukaryotes, prokaryotes) was pooled to identify those residues that are conserved (or almost conserved) among all classes.

Homology modelling

A homology model of the NBSs of SUR1 was constructed as described previously (de Wet et al. 2007b). Briefly, the structure of the ABC protein Sav1886 (Dawson & Locher, 2006, 2007) was used as a template. SUR1 consists of three sets of transmembrane domains (TMDs) and two NBDs, whereas Sav1866 contains a single TMD and NBD, which associate as a homodimer. For modelling, the sequences of TMD1, NBD1, TMD2 and NBD2 of SUR1 (residues 301–1581) were aligned with two consecutive copies of the Sav1866 monomer (1152 residues) by using the program Modeller v7 (Sali & Blundell, 1993; http://salilab.org/modeller/), of which the model with the lowest energy in a GROMACS (www.gromacs.org) energy minimization was chosen.

Nucleotide hydrolysis by NBD2

The C-terminus of maltose binding protein was fused to the N-terminus of the second nucleotide-binding domain of rat SUR1 (residues Val618 to Leu1004) using the (pMal-c2X vector system, New England Biolabs, Hitchin, UK) and expressed as a fusion protein, abbreviated here as NBD2. Site-directed mutagenesis was carried out according to the manufacturer’s instructions (QuickChange SDM kit, Stratagene, La Jolla, CA, USA). Wild-type (WT) and mutant proteins were purified as described (de Wet et al. 2007a).

ATPase activity was measured using a colorimetric assay for liberated inorganic phosphate (P<sub>i</sub>) at 37°C (de Wet et al. 2007a). Experimental repeats (n) refer to separate protein preparations. All protein preparations were assayed in duplicate. Values are means ± SEM. The Michaelis–Menten (MM) equation was fitted to concentration–activity relationships and used to obtain the K<sub>m</sub>. Turnover rate (nmol P<sub>i</sub> released s<sup>−1</sup> (nmol protein)<sup>−1</sup>) was calculated from V<sub>max</sub> values generated by fitting the MM equation to the data, using a molecular mass of 74 kDa for MBP-NBD2.

Electrophysiology

Kir6.2 (Genbank NM000525; E23 and I337) and rat SUR1 (Genbank L40624) were used. Site-directed mutagenesis of Kir6.2, synthesis of capped mRNA and preparation and injection of Xenopus laevis oocytes was performed as previously reported (Craig et al. 2008). All experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 and University of Oxford ethical guidelines. Oocytes were co-injected with ~2 ng of SUR1 mRNA and ~0.1 ng wild-type or mutant Kir6.2 mRNA. To simulate the heterozygous state, SUR1 was coexpressed with a 1:1 mixture of wild-type and mutant Kir6.2. For each batch of oocytes, all mutations were injected to enable direct comparison of their effects. Oocytes were maintained in Barth’s solution and studied 1–7 days after injection.

Macroscopic currents were recorded from giant inside-out patches. The pipette solution contained (mm): 140 KCl, 1.2 MgCl<sub>2</sub>, 2.6 CaCl<sub>2</sub>, 10 Hepes (pH 7.4 with KOH). The Mg-free internal (bath) solution contained (mm): 107 KCl, 1 K<sub>2</sub>SO<sub>4</sub>, 10 EGTA, 10 Hepes (pH 7.2 with KOH) and K<sub>2</sub>ATP, as indicated. The Mg-containing solution was made by adding 2 mm MgCl<sub>2</sub> to Mg-free solution, and using MgATP rather than ATP.

The macroscopic slope conductance was measured between −20 and −100 mV, in response to 3 s voltage ramps from −100 mV to +110 mV (holding potential −10 mV). To control for possible rundown when measuring nucleotide inhibition, the control conductance (G<sub>c</sub>) was taken as the mean of the conductance in control solution before and after nucleotide application. Nucleotide concentration–inhibition curves were fitted with a modified Hill equation:

\[ G/G_c = a + (1 - a)/(1 + ([X]/IC_{50})^h), \]  

where [X] is the concentration of nucleotide and IC<sub>50</sub> is the concentration of nucleotide that halves the maximum conductance (G<sub>c</sub>).
where \( G \) is the conductance in the presence of nucleotide, \([X]\) is the nucleotide concentration, \( IC_{50} \) is the nucleotide concentration at which inhibition is half-maximal, \( h \) is the slope factor (Hill coefficient) and \( a \) represents the fraction of unblocked current at saturating nucleotide concentration (\( a = 0 \) except where specified).

When measuring the relationship between nucleotide concentration and channel activation, we measured current amplitudes at \(-60 \text{ mV}\). Each test nucleotide concentration was interrupted by nucleotide-free solution and the control current \((I_c)\) was taken as the mean of the current in control solution before and after nucleotide application. The relationship between nucleotide activation and nucleotide concentration was fitted with eqn (2):

\[
\frac{I_x}{I_c} = 1 + \left( a \left( [X]/IC_{50}\right)^h \right) / \left( 1 + ([X]/IC_{50})^h \right)
\]

where \( I_x \) is the current in the presence of the test concentration of nucleotide, \( I_c \) is the current in nucleotide-free solution (obtained by averaging the current before and after application of the nucleotide), \([X]\) is the nucleotide concentration, \( EC_{50} \) is the nucleotide concentration at which activation is half-maximal, \( h \) is the slope factor (Hill coefficient) and \( a \) is the maximum level of current activation.

Single-channel currents were recorded at \(-60 \text{ mV}\) from inside-out patches and analysed using a combination of Clampfit (Molecular Devices, Sunnyvale, CA, USA), OriginLab Corp., Northampton, MA, USA) and in-house software (Craig et al. 2008). The open probability in the absence of ATP \((P_{o(0)}\) was determined from single-channel patches as the fraction of time spent in the open state for recordings of \(\sim 1 \text{ min}\) duration.

Data are given as means ± SEM. Significance was evaluated using Student’s t test.

**Results**

**Identification of conserved residues**

Amino acid sequences of 12 ABC transporters of the ABCC subfamily plus three additional eukaryotic (PgP, ABCA1, Tap1 + 2) and five additional prokaryotic ABC proteins (Sav1866, MsBA, BtuCD, ModBC, Hi1470/71) were aligned using the software ClustalX without manual curation. A representative sequence alignment is shown in Fig. 1. Twenty-three residues were found to be perfectly conserved among these sequences (see the online Supplemental Information, Supplemental Table 1). With four exceptions, all of these residues lie within regions of the protein known to be important for ATP binding and hydrolysis, such as the Walker A and B motifs, the signature sequence and the D-loop. The exceptions are three residues in NBD1 (SUR1 G735, A841 and G912) and a single residue, G1401, in NBD2. G1401 is equivalent to G735 in NBD1, and is located 16 positions downstream from the NBD2 Walker A catalytic lysine residue (Fig. 1A and B). The function of G1401 is unknown but the fact that it is invariant across all ABC transporters implies an important role.

In crystal structures of the NBDs of other ABC proteins, the residue equivalent to SUR1-G1401 does not interact directly with the nucleotide, the transmembrane regions or the opposite NBD. Instead, the glycines are located on the outside of the domains and are exposed to the cytoplasm. Homology modelling of the NBDs of SUR1, based on the structure of Sav1866, places G1401 in a similar position, far from the nucleotide-binding sites (Fig. 1B and C).

To determine the function of G1401, we examined the effect of mutating this residue on both the ATPase activity of SUR1 and its ability to act as a regulator of Kir6.2. We chose to mutate the residue to arginine, as this mutation has been found in a patient with congenital hyperinsulinism (Stanley et al. 2004).

**ATPase activity**

ATPase activity was measured on isolated NBD2, fused to the maltose-binding protein to enhance solubility (de Wet et al. 2007a). Wild-type NBD2 hydrolysed ATP with a turnover rate \((k_{cat})\) of 0.064 s\(^{-1}\) and a \(K_m\) of 0.30 mm (Fig. 2 and Table 1), values similar to those reported previously (Masia et al., 2005; de Wet et al. 2007a; de Wet et al. 2010). Mutation of glycine 1401 to arginine decreased \(k_{cat}\) by \(\sim 25\%\) without altering the \(K_m\) (Fig. 2 and Table 1). Inhibition by BeF was unaltered by the G1401R mutation, which suggests the affinity for P\(_i\) is not affected (Supplemental Fig. 1A). Product-dependent inhibition of ATPase activity by MgADP \((K_{i(MgADP)}\) was also unchanged (Supplemental Fig. 1B), indicating MgADP binding is unimpaired.

**ATP inhibition of \(K_{ATP}\) currents**

To examine the effect of the G1401R mutation on the nucleotide sensitivity of the \(K_{ATP}\) channel, we coexpressed wild-type or mutant SUR1 with Kir6.2 in *Xenopus* oocytes and applied nucleotides to the intracellular surface of inside-out membrane patches. There was no obvious difference in current magnitude in patches from oocytes expressing wild-type or G1401R channels: the mean current amplitude following excision was 1.8 ± 0.5 nA \((n=8, \text{Fig. 3A})\) for wild-type and 2 ± 0.8 nA \((n=15, \text{Fig. 3B})\) for G1401R channels. Thus the mutation has little effect on channel expression levels.

In the absence of Mg\(^{2+}\), ATP is suggested to influence \(K_{ATP}\) channel activity only via binding to Kir6.2 (Gribble et al. 1998). Figure 3C shows that
Figure 1. Invariant residues in the NBDs of ABC proteins
A, alignment of the NBDs of various ABC proteins showing the invariant glycine residue that lies C terminal to the Walker A motif. Abbreviations for the eukaryotic proteins are: MRP, multidrug resistance related protein; CFTR, cystic fibrosis transmembrane conductance regulator; PgP, P-glycoprotein; Tap, transporter associated with antigen processing. MsbA, ModC, BtuCD and Sav1866 are bacterial ABC proteins. R, rat; h, human. B and C, homology model of the human SUR1-NBD1 and SUR1-NBD2 dimer (B) and SUR1-NBD2 alone (C). The Walker A motif is shown in red, the Walker B motif in purple, and the ABC signature sequence in orange. G1401 is shown as a sphere. NBD1 is shown in silver (right) and NBD2 in blue (left). The Mg^{2+} atom is shown as a white sphere and ATP (in ball-and-stick format) in green.
the ATP concentration–inhibition relation for G1401R channels deviated from that of wild-type at nucleotide concentrations greater than 1 μM, becoming progressively less ATP sensitive. At 0.1 mM ATP, wild-type $K_{\text{ATP}}$ channels were fully blocked but a significant G1401R current remained. This suggests either that the SUR1 mutation influences ATP binding at Kir6.2, or that ATP binding to SUR1 in the absence of Mg$^{2+}$ leads to channel inhibition. One way in which SUR1 mutations can influence ATP block at Kir6.2 is by increasing the intrinsic channel open probability (i.e. that in the absence of ligand), which leads to a secondary decrease in ATP inhibition (Trapp et al. 1998; Proks et al. 2007). However, there was no difference in the open probability of wild-type (0.19 ± 0.05, $n = 6$) and G1401R (0.21 ± 0.05; $n = 6$) channels in nucleotide-free solution: thus the reduced ATP sensitivity is not a consequence of changes in open probability.

When Mg$^{2+}$ was present, mutant channels were ∼2.5-fold less sensitive to ATP than wild-type channels, the IC$_{50}$ being 16 μM and 40 μM ATP for wild-type and mutant channels, respectively (Fig. 3D and Table 2). The amount of current remaining unblocked at 1 mM MgATP concentrations also increased, to ∼3%, which is similar to that found for G1401R channels in the absence of Mg$^{2+}$ (Table 2). ATP concentration–response relations for each channel type in the presence and absence of Mg$^{2+}$ are compared in Supplemental Fig. 2. It is clear that for wild-type channels Mg$^{2+}$ causes a parallel shift in ATP sensitivity along the concentration axis. In contrast, Mg$^{2+}$ produces a marked reduction in ATP inhibition of G1401R channels at a concentration of 10 μM ATP, but has no significant effect at higher MgATP concentrations.

### Channel activation by MgADP

The decrease in ATP inhibition in the presence of Mg$^{2+}$ produced by the G1401R mutation might be explained if Mg-nucleotide activation is enhanced, as is found for some other ND mutations (Proks et al. 2005; Babenko et al. 2006). To determine if this is the case, we next measured the ability of 100 μM MgADP to stimulate channel activity in the presence and absence of 100 μM MgATP. Unexpectedly, G1401R channels were not activated by MgADP (Fig. 4A): instead MgADP produced a substantial block of the channel. Similarly, MgADP was unable to reverse inhibition produced by MgATP: indeed, the block was actually greater in the presence of both MgADP and MgATP. This suggests that the G1401R mutation might impair MgADP activation.

We therefore measured ADP concentration–response curves, in the presence and absence of Mg$^{2+}$, for G1401R channels (Fig. 4B). As previously reported for wild-type channels, ADP inhibited G1401R channels in the absence of Mg$^{2+}$. The IC$_{50}$ was 42 ± 3 μM ($n = 5$), which is not dissimilar to that found for wild-type channels (64 ± 1 μM, Dabrowski et al. 2004; 62 μM, Proks et al. 2010). The extent of ADP block of G1401R channels was less in the presence of Mg$^{2+}$, the IC$_{50}$ being 91 ± 1 μM ($n = 9$). Nevertheless, this result is in dramatic contrast to what is observed for wild-type channels, where MgADP causes a marked stimulation of channel activity (Nichols, 2006; Proks et al. 2010). The difference can be explained if the G1401R mutation impairs MgADP activation of the channel. The small shift in the ADP concentration–response curve in the presence of Mg$^{2+}$ may suggest that activation is not fully abolished, and that which remains is able to partially mask nucleotide inhibition at Kir6.2.

To determine the extent of Mg-nucleotide activation more precisely, we coexpressed SUR1-G1401R with a mutant Kir6.2 subunit containing a mutation that abolishes ATP inhibition at Kir6.2. We selected the G334D mutation, which abolishes ATP block completely (Drain et al. 1988; Masia et al. 2007). This strategy enables the stimulatory effects of Mg-nucleotides to be studied in

---

**Table 1. ATPase activities and kinetic constants**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Turnover rate (s$^{-1}$)</th>
<th>$V_{\text{max}}$ (nmol Pi min$^{-1}$ mg$^{-1}$)</th>
<th>$K_{m}$ (μM)</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBD2-WT</td>
<td>0.064 ± 0.01</td>
<td>51.7 ± 5</td>
<td>0.30 ± 0.02</td>
<td>4</td>
</tr>
<tr>
<td>NBD2-G1401R</td>
<td>0.049 ± 0.05</td>
<td>39.7 ± 3.6$^{∗∗}$</td>
<td>0.34 ± 0.05</td>
<td>4</td>
</tr>
</tbody>
</table>

WT, wild-type. Significance against NBD2-WT: $^{∗∗}P < 0.01$.

---

**Figure 2. ATPase activity of NBD2 is decreased by the G1401R mutation**

ATPase activity of isolated MBP-NBD2. Wild-type: circles, $n = 4$. G1401R: triangles, $n = 4$. The lines are the best fit of the Michaelis–Menten equation to the mean data.
isolation from their inhibitory effects at Kir6.2 (Proks et al. 2010). Figure 5A shows that half-activation (EC$_{50}$) of Kir6.2-G334D/SUR1 channels by MgADP occurred at 37 ± 15 μM, somewhat greater than that found previously (8 μM, Proks, et al 2010). MgADP activation was markedly decreased by the SUR1-G1401R mutation – to 7% of that for wild-type SUR1 at 1 mM MgADP. Similar results were found for MgATP, where nucleotide activation was also only 7% of wild-type SUR1 (at 10 mM MgATP, Fig. 5B).

These results indicate that both MgATP and MgADP activation are largely inhibited by the G1401R mutation.

Because nucleotide activation is largely abolished, the data also suggest that the shift in the ADP and ATP inhibition curves of G1401R channels produced by Mg$^{2+}$ may not in fact reflect channel activation. To determine if this separate action of Mg-nucleotides involves the ATP-binding sites of SUR1, we mutated the Walker A lysines in both NBD1 and NBD2 to alanine. 

**Figure 3.** ATP inhibition of wild-type and G1401R channels in the absence and presence of Mg$^{2+}$

A and B, $I_{\text{KATP}}$ currents recorded in response to voltage ramps from −110 to +110 mV in an inside-out patch excised from oocytes expressing wild-type (A) or Kir6.2/SUR1-G1401R (B) channels. The dashed line indicates the zero current level. The bar indicates application of 10 μM ATP.

C and D, mean relationships between [ATP] and $I_{\text{KATP}}$ conductance ($G$), expressed relative to that in the absence of nucleotide ($G_c$), for wild-type ($\bullet$, $n = 10$), and Kir6.2/SUR1-G1401R ($\circ$, $n = 8$) channels in the absence of Mg$^{2+}$ (C); or for wild-type ($\bullet$, $n = 8$) or Kir6.2/SUR1-G1401R ($\circ$, $n = 21$) currents in the presence of Mg$^{2+}$ (D). The lines are drawn to eqn (1) with the following parameters. C: wild-type, IC$_{50}$ = 4.7 μM, h = 1.2; G1401R, IC$_{50}$ = 7.3 μM, h = 0.8, a = 0.011. D: wild-type, IC$_{50}$ = 12 μM, h = 1.0; G1401R, IC$_{50}$ = 27 μM, h = 1.1, a = 0.03.
Table 2. Nucleotide block of wild-type and mutant channels

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-type (G1401R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$IC_{50}$ (Mg$^{2+}$-free)</td>
<td>$5.6 \pm 0.7 \mu M$</td>
</tr>
<tr>
<td>(solution)</td>
<td>($n = 10$)</td>
</tr>
<tr>
<td>$IC_{50}$ (Mg$^{2+}$ solution)</td>
<td>$16 \pm 2 \mu M$</td>
</tr>
<tr>
<td>(solution)</td>
<td>($n = 8$)</td>
</tr>
<tr>
<td>Current remaining in 3 mM MgATP</td>
<td>$0.6 \pm 0.3%$</td>
</tr>
<tr>
<td>(n = 8)</td>
<td>($n = 21$)</td>
</tr>
</tbody>
</table>

WT, wild-type. $IC_{50}$, half-maximal block produced by ATP in the absence or presence of Mg$^{2+}$. *** $P < 0.001$

Discussion

Functional effects

Our data reveal that the G1401R mutation causes a small reduction in the turnover rate of ATP hydrolysis at NBD2, without affecting the $K_m$. The latter finding suggests ATP binding is unaffected by the mutation. Because MgADP inhibition of ATPase activity was not impaired, it appears that MgADP binding to NBD2 is also unaffected by the mutation. Despite the lack of an effect on Mg-nucleotide binding, and the relatively small effect on MgATP hydrolysis, activation of G1401R channels by both MgATP and MgADP was strongly reduced. This suggests that the mutation impairs the ability of bound Mg-nucleotide at NBS2 to enhance channel activity. Our biochemical studies were carried out on isolated NBDs and it is, of course, possible that the mutation alters nucleotide binding and/or hydrolysis in the whole K$\text{ATP}$ channel complex. Nevertheless, given the location of the mutation, far distant from the NBSs, it seems plausible that the mutation lacks an effect on nucleotide binding/hydrolysis in the octameric complex, as it does in the isolated NBD. With this caveat, G1401R is the first mutation to be shown to uncouple Mg-nucleotide binding/hydrolysis at SUR1 from stimulation of channel activity. It seems likely that it influences the mechanism by which the presence of MgADP at NBS2 of SUR1 is translated into opening of the Kir6.2 pore.
Figure 5. Mg-nucleotide activation of wild-type and mutant K\textsubscript{ATP} channels

A and B, mean relationships between [MgADP] (A) or [MgATP] (B) and K\textsubscript{ATP} conductance (G) expressed relative to that in the absence of nucleotide (G\textsubscript{c}), for Kir6.2-G334D/SUR1 (o, n = 5) and Kir6.2-G334D/SUR1-G1401R (*) channels. The lines through the G1401R data are drawn by eye. The lines through the wild-type data are drawn to eqn (2) with the following parameters: A: EC\textsubscript{50} = 38 \mu M, h = 2, a = 10; B: EC\textsubscript{50} = 196 \mu M, h = 1.6, a = 4.6.

Structural considerations

Homology modelling of the NBSs of SUR1 places G1401 on the outer surface of the protein, on the cytoplasmic side of the membrane, distant from the nucleotide-binding site. Thus it would be unable to influence nucleotide binding or hydrolysis directly, which may explain why its mutation has little influence on either process. Instead, its position argues it might be located in vicinity of Kir6.2 or a neighbouring SUR1 monomer. This might explain why it appears to affect the mechanism by which conformational change induced by nucleotide binding and/or hydrolysis is translated in pore opening.

Glycine is unique among the natural amino acids in allowing for a far wider range of protein backbone conformations, and it is often located at the ends of secondary structure elements or in turns and loops. The available crystal structures of ABC transporter NBDs confirm such a location for the conserved glycines equivalent to SUR1-G1401. In the bacterial exporter Sav1866 (Dawson & Locher, 2006; 2007), the eukaryotic P-gp (Aller et al. 2009), and the isolated NBD1 of closely related the ABCC protein MRP1 (Ramaen et al. 2006), the glycine located in the position equivalent to G1401R lies at the beginning of a six to seven residue-long \beta-strand, which ends at another conserved glycine (G1407 in SUR1). It seems likely that the backbone flexibility of a glycine is required at position 1401 of SUR1 to connect two extended strands at a particular angle. Mutation of G1401 to another residue might influence the conformation of the \beta-strand it lies adjacent to, and thereby influence the mechanism by which MgADP occupancy at NBS2 is transduced to Kir6.2.
Effects on ADP block

$K_{ATP}$ channel activity is determined by the balance between the inhibitory effects of nucleotides (such as ATP and ADP) at Kir6.2, and the stimulatory effects of Mg-nucleotides that are mediated via SUR1. Our results show that, although MgADP does not activate the mutant channel, Mg$^{2+}$ produces a small shift in the ADP concentration–inhibition relationship of G1401R channels to higher nucleotide concentrations. This is in marked contrast to wild-type channels, where addition of Mg$^{2+}$ converts the response to ADP from an inhibitory to a stimulatory one (Proks et al. 2010).

Effects on ATP block in the absence of Mg$^{2+}$

Why G1401R channels are less sensitive to higher concentrations of ATP in the absence of Mg$^{2+}$ is matter of speculation. One possibility is that the mutation impairs ATP binding to Kir6.2. It is well documented that SUR1 enhances the efficacy of ATP inhibition, the $IC_{50}$ decreasing from $\sim 100 \mu M$ for Kir6.2ΔC to 10 $\mu M$ for Kir6.2/SUR1, when measured in the absence of Mg$^{2+}$ (Tucker et al. 1997). How SUR1 mediates this effect is unclear—it has been suggested that it exerts an allosteric action on the structure of the ATP-binding site on Kir6.2, or even contributes residues to the binding site. Thus the G1401R mutation may somehow disrupt this interaction. This seems plausible given the predicted location of G1401R in the protein structure. However, if this was the case, we would expect that, in the absence of Mg$^{2+}$, ADP would also block G1401R channels less strongly than wild-type—which is not the case. This would therefore support the suggestion that G1401R uncouples communication between the SUR1 and Kir6.2 subunits, without directly affecting nucleotide binding to either.

Another hypothesis is that nucleotide binding to SUR1 has a direct inhibitory effect on Kir6.2 that is impaired by the G1401R mutation. It is known that ATP binds to NS1 of SUR1 with high affinity in the absence of Mg$^{2+}$ (Ueda et al. 1997). If this binding leads to channel inhibition, the G1401R mutation might uncouple nucleotide binding at this site from changes in channel activity, as it does at NSB2. Alternatively, ATP binding to NS1 (in the absence of Mg$^{2+}$) might stimulate channel activity and the G1401R mutation might facilitate this effect. Against both these ideas is the fact that high-affinity 8-azido-[32P]ATP binding to NS1 is inhibited by mutation of the NBD1 Walker A lysine (Ueda et al. 1997) whereas G1401R channels were still blocked less than wild-type when both Walker A lysines were mutated. Nevertheless, the different structures of 8-azido-ATP and ATP leave the possibility that the mutation does not totally prevent binding of ATP. Detailed studies of equilibrium ATP binding are needed to exclude the involvement of nucleotide interactions with SUR1 in the lower Mg-free ATP sensitivity of G14101R channels.

Why does Mg$^{2+}$ reduce the ATP sensitivity of G1401R channels?

Addition of Mg$^{2+}$ produces a large reduction in ATP inhibition of G1401R channels at nucleotide concentrations between 1 and 100 $\mu M$. This cannot be a consequence of MgATP stimulation of channel activity, as our data indicate this is almost abolished by the G1401R mutation and non-existent at 10 $\mu M$ MgATP (when measured with the Kir6.2-G334D mutation). Similarly, Mg$^{2+}$ reduces ADP inhibition at nucleotide concentrations at which no activation is present. Growing evidence suggests that the small shift produced in the wild-type ATP concentration–inhibition curve by Mg$^{2+}$ is also not due to nucleotide activation because it occurs at MgATP concentrations at which the stimulatory effects are minimal (10 $\mu M$, Fig. 5 and Proks et al. 2010). The ability of G1401R mutation to enhance this process while abolishing Mg-nucleotide activation suggests that the two processes are distinct.

What mechanism might underlie the shift in the ATP concentration–inhibition curve produced by Mg$^{2+}$? Again, we can only speculate. It is clear that it is seen for both MgATP and MgADP, that it requires Mg$^{2+}$, that it is initiated at nucleotide concentrations around 1 $\mu M$, and that it is abolished by mutation of one or both of the Walker A lysines in SUR1. Thus the simplest explanation is that it is produced by Mg-nucleotide binding to the NBSs to SUR1. Evidence presented here suggests that it is not mediated by nucleotide binding to NSB2. The current consensus is that occupancy of NSB2 by MgADP stimulates channel activity: our data indicate that this effect is abolished by the G1401R mutation whereas the Mg-dependent shift in ATP sensitivity is not. Thus we favour the idea that MgATP or MgADP binding to NS1 reduces ATP inhibition at Kir6.2. The nucleotide concentrations at which these effects occur are consistent with the $K_i$ for ATP (4 $\mu M$) and ADP (26 $\mu M$) inhibition of 8-azido-[32P]ATP binding to NS1 of SUR1 (Matsuo et al. 2000). As no increase in channel activity is observed for ATP-insensitive $K_{ATP}$ channels (e.g. those carrying the Kir6.2 mutation G334D) at low (10 $\mu M$) ATP concentrations, we favour the idea that nucleotide occupancy of NS1 does not increase channel activity $per se$. Rather, it reduces the ability of ATP to block Kir6.2, by affecting either ATP binding or the transduction of ATP binding into a decrease in channel open probability.

In conclusion, we have shown that residue G1401 plays a crucial role in translating MgADP occupancy of NSB2 of SUR1 into increased activity of the $K_{ATP}$ channel. It may also be a useful tool to dissect out how other nucleotide interactions with SUR1 modulate the function of Kir6.2.
Effects of the SUR1-G1401R mutation on $K_{ATP}$ channel function

References


© 2012 The Authors. The Journal of Physiology © 2012 The Physiological Society


**Author contributions**

H.d.W. and F.M.A conceived and designed the experiments. H.d.W., K.S., J.A., N.A. and M.L. collected, analysed and interpreted the data. F.M.A., H.d.W., J.A. and M.S. participated in conception and design and wrote the manuscript. All authors approved the final version of the manuscript.

**Acknowledgements**

We thank the Wellcome Trust (076436/Z/05/Z and 081188/A/06/Z), the Royal Society, and the European Union EuroDia-(LSHM-CT-2006-518153) for support. H.d.W. is an EFSD/Lilly research fellow. F.M.A. is a Royal Society Research Professor.