# The Kir6.2-F333I mutation differentially modulates $K_{ATP}$ channels composed of SUR1 or SUR2 subunits

Paolo Tammaro and Frances Ashcroft

*Department of Physiology, Anatomy and Genetics, Parks Road, Oxford OX1 3PT, UK*

**Mutations in Kir6.2, the pore-forming subunit of the $K_{ATP}$ channel, that reduce the ability of ATP to block the channel cause neonatal diabetes. The stimulatory effect of MgATP mediated by the regulatory sulphonylurea receptor (SUR) subunit of the channel may also be modified. We compared the effect of the Kir6.2-F333I mutation on $K_{ATP}$ channels containing SUR1, SUR2A or SUR2B. The open probability of Kir6.2/SUR1 channels, or a C-terminally truncated form of Kir6.2 expressed in the absence of SUR, was unaffected by the mutation. However, that of Kir6.2/SUR2A and Kir6.2/SUR2B channels was increased. In the absence of $Mg^{2+}$, ATP inhibition of all Kir6.2-F333I/SUR channel types was reduced, although SUR1-containing channels were reduced more than SUR2-containing channels. These results suggest F333 is involved in differential coupling of Kir6.2 to SUR1 and SUR2. When $Mg^{2+}$ was present, ATP *blocked* SUR2A channels but *activated* SUR2B and SUR1 channels. Activation by MgGDP (or MgADP) was similar for wild-type and mutant channels and was independent of SUR. This indicates Mg-nucleotide binding to SUR and the transduction of binding into opening of the Kir6.2 pore are unaffected by the mutation. The data further suggest that MgATP hydrolysis by the nucleotide-binding domains of SUR1 and SUR2B, but not SUR2A, is enhanced by the F333I mutation in Kir6.2. Taken together, our data suggest the region of the C terminus within which F333 lies is involved in more than one type of functional interaction with SUR, and that F333 interacts differentially with SUR1 and SUR2.**

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



ATP-sensitive $K^+$ ($K_{ATP}$) channels serve as metabolic sensors, coupling cell metabolism to electrical activity. They thereby regulate insulin secretion from pancreatic $\beta$-cells, and transmitter release from brain neurones, modulate vascular tone, and participate in the response to ischaemic stress in brain and heart (Seino & Miki, 2003).

The $K_{ATP}$ channel comprises Kir6.x and SUR subunits (Shi & Makielski, 2005). In most tissues, Kir6.2 forms a central tetrameric pore. It is surrounded by four regulatory SUR subunits, which modulate the opening and closing (gating) of the channel (Shyng & Nichols, 1997). The SUR2A isoform is found in cardiac myocytes (Inagaki *et al.* 1996; Morrissey *et al.* 2005), SUR2B in neurones and both vascular and non-vascular smooth muscles (Isomoto *et al.* 1996), and SUR1 in most other tissues, including $\beta$-cells and many neurones (Aguilar-Bryan *et al.* 1995; Shi *et al.* 2005).

The most important modulators of $K_{ATP}$ channel gating are adenine nucleotides: ATP closes the channel by binding to Kir6.2, whereas interaction of Mg-nucleotides (MgATP, MgADP) with the nucleotide-binding domains (NBDs) of SUR stimulates channel activity and reverses channel inhibition by ATP (Tucker *et al.* 1997; Matsuo *et al.* 2005). Nucleotide modulation enables pancreatic $K_{ATP}$ channels to regulate insulin secretion in response to changes in plasma glucose, and mediates opening of cardiac and neuronal $K_{ATP}$ channels in response to ischaemia.

Heterozygous activating mutations in both Kir6.2 and SUR1 cause neonatal diabetes (ND), by reducing the sensitivity of the $K_{ATP}$ channel to inhibition by MgATP (Ashcroft, 2005; Babenko *et al.* 2006). Some mutations produce a more severe clinical phenotype in which developmental delay, epilepsy and muscle weakness accompany neonatal diabetes (Hattersley & Ashcroft, 2005). However, although Kir6.2 is expressed in heart and nonvascular smooth muscles (Shi *et al.* 2005), no clinical symptoms have been reported in these tissues in patients carrying Kir6.2 mutations. This may be because the functional effects of the Kir6.2 mutation are modulated by the type of SUR subunit, being more severe for SUR1 than SUR2A (Tammaro *et al.* 2006). The effects of coexpression with SUR2B have not been reported to date.

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ND mutations in Kir6.2 enhance $K_{ATP}$ channel activity by decreasing ATP inhibition at Kir6.2 (Proks *et al.* 2004; Ashcroft, 2005). They do so in a variety of ways. Some mutations appear to decrease ATP binding and/or transduction at Kir6.2. Other mutations act indirectly, by increasing the open state stability of the channel which, in turn, decreases $K_{ATP}$ channel block by ATP (Trapp *et al.* 1998; Enkvetchakul *et al.* 2001). In addition, Kir6.2 mutations can affect the way in which SUR couples to Kir6.2 (Proks *et al.* 2005).

Previous studies have shown that SUR modulates Kir6.2 in at least three ways: it modifies the gating of wild-type Kir6.2, enhances the ATP sensitivity, and confers sensitivity to the stimulatory effects of Mg-nucleotides (which act via SUR). These effects can be differentiated by comparing the properties of Kir6.2/SUR channels with those of Kir6.2ΔC, a variant of Kir6.2 that lacks the last 26–36 amino acids and that, unlike Kir6.2, does not require SUR for trafficking to the surface membrane (Tucker *et al.* 1997; Zerangue *et al.* 1999). To date, ND mutations have been shown to affect the way in which SUR1 modulates the gating of Kir6.2, and to increase channel activation by both Mg-nucleoside diphosphates and Mg-nucleoside triphosphates (Proks *et al.* 2005).

We previously showed that the ND mutation Kir6.2-F333I produces a marked decrease in the ATP sensitivity of Kir6.2/SUR1 channels (Tammaro *et al.* 2005). Here, we compare the functional effects of this mutation on $K_{ATP}$ channels composed of different SUR isoforms with that of Kir6.2ΔC. We show that the F333I mutation affects channel gating only when SUR2, but not SUR1, is present in the $K_{ATP}$ channel complex, indicating that residue F333 is involved in coupling to SUR2 but not SUR1. Unlike ND mutations studied earlier (Tammaro *et al.* 2006), the extent of activation by Mg-nucleotide *di*phosphates is unaffected by the F333I mutation: thus it does not alter nucleotide binding/transduction. Instead, the mutation dramatically enhances activation of SUR1 and SUR2B, but not SUR2A, channels by MgATP. This suggests that the Kir6.2 mutation affects ATP hydrolysis at SUR and that this effect is greatest for SUR1 and least for SUR2A. These effects combine to alter the response of the whole-cell currents through F333I mutant channels to metabolic inhibition.

## Methods

### Molecular biology

Human Kir6.2 (GenBank D50581) or Kir6.2-F333I and rat SUR1 (GenBank L40624), SUR2A (D83598) and SUR2B (D86038) were used in this study. Site-directed mutagenesis of Kir6.2 and preparation of mRNA were performed as previously described (Gribble *et al.* 1997*a*).

### Oocyte preparation

Female *Xenopus laevis* were anaesthetized with MS222 ($2\,g\,l^{-1}$ added to the water). One ovary was removed via a mini-laparotomy, the incision was sutured, and the animal was allowed to recover. Subsequently, animals were operated on for a second time, but under terminal anaesthesia. Immature stage V–VI oocytes were incubated for 60 min with $1\,mg\,ml^{-1}$ collagenase (type V; Sigma) and manually defolliculated. All procedures were carried out in accordance with UK Home Office Legislations and the University of Oxford ethical guidelines. Oocytes were coinjected with ∼0.8 ng wild-type or mutant Kir6.2 mRNA and ∼4 ng of mRNA encoding SUR. The final injection volume was 50 nl per oocyte. Isolated oocytes were maintained in Barth's solution and studied 1–4 days after injection.

### Electrophysiology

Whole-cell currents were recorded from intact oocytes using a two-electrode voltage clamp (Gribble *et al.* 1997*a*) in response to voltage steps of ±20 mV from a holding potential of −10 mV, filtered at 1 kHz and digitized at 4 kHz. Oocytes were perfused with a solution containing (mM): 90 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, and 5 Hepes (pH 7.4 with KOH). Metabolic inhibition was produced by 3 mM sodium azide.

Macroscopic currents were recorded from giant inside-out patches using an EPC10 amplifier (List Medical Electronics, Darmstadt, Germany) controlled with Pulse v8.74 software (Heka Electronik, Lambrecht, Germany). Macroscopic currents were elicited by 3 s voltage ramps from −110 to +100 mV (holding potential, 0 mV), filtered at 0.5 kHz and digitized at 1 kHz. The pipette solution contained (mM): 140 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 10 Hepes (pH 7.4 with KOH). The $Mg^{2+}$-free internal (bath) solution contained (mM): 107 KCl, 1 $K_2SO_4$, 10 EGTA, 10 Hepes (pH 7.2 with KOH) and nucleotides as indicated. The $Mg^{2+}$-containing internal solution was the same as the $Mg^{2+}$-free solution but with the addition of 2 mM $MgCl_2$ and Mg-nucleotides (instead of $K_2$-nucleotides) as indicated. Experiments were conducted at 20–22°C. Solutions were changed using a local perfusion system consisting of tubes of ∼200 $\mu$m diameter into which the tip of the patch pipette was inserted.

Nucleotide concentration–inhibition curves were fit with the Hill equation:

$$\frac{G}{G_c} = \frac{1}{1 + ([\text{nucleotide}]/\text{IC}_{50})^h} \qquad (1)$$

where [nucleotide] is the nucleotide concentration, $IC_{50}$ is the nucleotide concentration at which inhibition is half-maximal and $h$ is the slope factor (Hill coefficient).

Concentration–activation curves were fit with:

$$\frac{G}{G_0} = \frac{1 - A}{1 + (EC_{50}/[\text{nucleotide}])^j} + A \qquad (2)$$

where $EC_{50}$ is the nucleotide concentration at which activation is half maximal, $A$ is the maximum level of activation, and $j$ is the slope factor.

Single-channel currents were measured at −60 mV, filtered at 5 kHz and digitized at 20 kHz. Unitary amplitude and channel open probability ($P_o$) were measured from the Gaussian fit to all-points amplitude histograms of tracts of current of 30–90 s duration.

Data were analysed with in-house routines developed in the IgorPro (Wavematrics, OR, USA) platform. Data are given as means ± S.E.M. Statistical significance was evaluated using a two-tailed Student $t$ test, and $P < 0.05$ was taken to indicate a significant difference.

## Results

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

Interaction of adenine nucleotides with SUR1, but not with Kir6.2, is $Mg^{2+}$ dependent (Gribble *et al.* 1998). Thus, to isolate the effects of ATP on Kir6.2 we performed experiments in $Mg^{2+}$-free solution. In the absence of $Mg^{2+}$, all three channel types were less sensitive to inhibition by ATP than wild-type channels (Fig. 1*A*, Table 1). Half-maximal block ($IC_{50}$) was produced by 208, 128 and 119 $\mu$M ATP for Kir6.2-F333I/SUR1, Kir6.2-F333I/SUR2A and Kir6.2-F333I/SUR2B channels, respectively (Fig. 1*B*; Table 1). These values are markedly greater than those found for wild-type channels (Table 1). However, the reduction in ATP sensitivity is significantly less when Kir6.2-F333I is coexpressed with SUR2A or SUR2B (∼12-fold) than with SUR1 (∼20-fold).

Mutations that impair channel inhibition by ATP in the absence of $Mg^{2+}$ may do so by reducing ATP binding, impairing the mechanism that transduces ATP binding into channel closure, or by enhancing the stability of the channel open state (Proks *et al.* 2004; Ashcroft, 2005). The intrinsic open probability ($P_{o(0)}$) of the channel, i.e. that measured in the absence of ATP, is not different for Kir6.2/SUR1 and Kir6.2-F333I/SUR1 channels, indicating the mutation does not affect the open state stability of SUR1-containing channels (Fig. 2, Table 2). In contrast, the $P_{o(0)}$ values of Kir6.2-F333I/SUR2A (0.71) and of Kir6.2-F333I/SUR2B (0.68) were greater than that of Kir6.2/SUR2A (0.40) or Kir6.2/SUR2B (0.41) (Fig. 2, Table 2). The larger $P_{o(0)}$ values will, indirectly, decrease channel inhibition by ATP (Proks *et al.* 2004). This is because an increase in $P_{o(0)}$ shifts the gating equilibrium towards the open state in both the absence and presence of ATP. The latter results in reduced $K_{ATP}$ current inhibition by the nucleotide (Trapp *et al.* 1998; Fan & Makielski, 1999; Enkvetchakul *et al.* 2000, 2001).

Because the ATP sensitivity of SUR2 channels is greater than that of SUR1 channels, despite the increased $P_{o(0)}$, it appears that ATP binding is not affected as severely when Kir6.2-F333I is coexpressed with SUR2A or SUR2B as with SUR1. The SUR subunit enhances the ATP sensitivity of Kir6.2 by an unknown mechanism (Tucker *et al.* 1997). For wild-type channels this enhancement is similar for SUR1 and SUR2 (in the absence of $Mg^{2+}$, Table 1). The difference in ATP sensitivity we observe thus suggests that SUR2 enhances the ATP sensitivity of Kir6.2-F333I more effectively than SUR1, and thus that F333I is involved in differential coupling of SUR1 and SUR2 to Kir6.2.

Previous studies have shown that some ND mutations in Kir6.2 only influence $P_{o(0)}$ when SUR is present (Tammaro *et al.* 2005, 2006; Proks *et al.* 2006). To test if this is also true of the F333I mutation, we used a C-terminally truncated form of Kir6.2 (Kir6.2ΔC) that expresses in the absence of SUR (Tucker *et al.* 1997). The $P_{o(0)}$ of Kir6.2ΔC-F333I ($0.027 \pm 0.011$; $n = 5$) was not significantly different from that of wild-type Kir6.2ΔC ($0.029 \pm 0.010$; $n = 5$). This

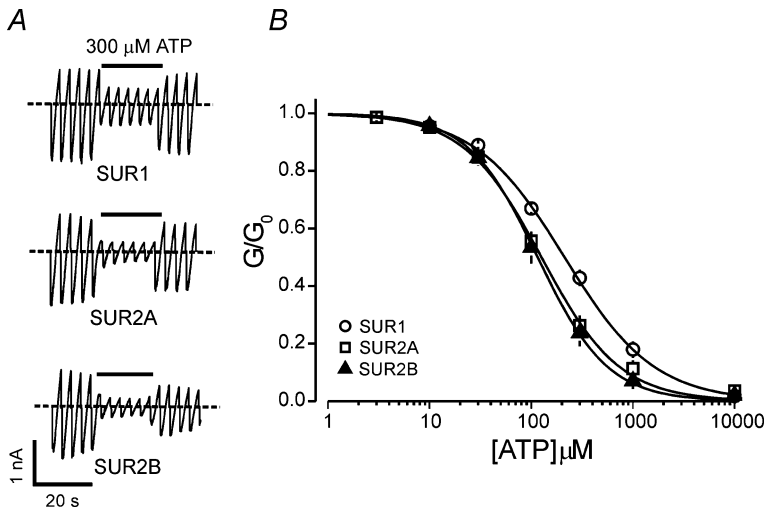


**Figure 1. ATP sensitivity in the absence of $Mg^{2+}$**
*A*, currents recorded in patches excised from *Xenopus* oocytes coexpressing Kir6.2-F333I and SUR1, SUR2A or SUR2B, as indicated, in response to voltage ramps from −110 to +100 mV. The dotted line indicates the zero current level. ATP (300 $\mu$M) was applied as indicated. *B*, mean relationship between [ATP] and $K_{ATP}$ conductance ($G$), expressed relative to the conductance in the absence of nucleotide ($G_0$), for channels containing the indicated SUR subunits. The curves are the best fit of eqn (1) to the data with $IC_{50}$ values of 198 $\mu$M (○, Kir 6.2-F333I/SUR1), 115 $\mu$M (□, Kir 6.2-F333I/SUR2A) and 116 $\mu$M (▲, Kir 6.2-F333I/SUR2B).

**Table 1. ATP sensitivity of Kir6.2/SUR and Kir6.2-F333I/SUR channels**

| | $Mg^{2+}$-free | | 2 mM $Mg^{2+}$ | | |
|---|---|---|---|---|---|
| Sur subtype | $IC_{50}$ ($\mu$M) | Slope factor | $IC_{50}$ or $EC_{50}$($\mu$M)[a] | Slope factor | *A* |
| Kir6.2/SUR | | | | | |
| SUR1 | 8.2 ± 2.0 ($n = 6$) | 1.3 ± 0.09 ($n = 6$) | 16 ± 3 ($n = 5$) | 1.10 ± 0.05 ($n = 5$) | — |
| SUR2A | 10.1 ± 1.8 ($n = 5$) | 1.2 ± 0.05 ($n = 5$) | 22 ± 3 ($n = 5$) | 1.14 ± 0.09 ($n = 5$) | — |
| SUR2B | 7.1 ± 2.5 ($n = 4$) | 1.4 ± 0.10 ($n = 4$) | 78 ± 11 ($n = 8$) | 1.30 ± 0.17 ($n = 8$) | — |
| Kir6.2-F333I/SUR | | | | | |
| SUR1 | 208 ± 12 ($n = 7$) | 1.02 ± 0.05 ($n = 7$) | 113 ± 8[b] ($n = 6$) | 2.18 ± 0.27 ($n = 6$) | 4.6 ± 0.17 ($n = 6$) |
| SUR2A | 128 ± 9 ($n = 6$) | 1.14 ± 0.11 ($n = 6$) | 541 ± 81[c] ($n = 5$) | 0.62 ± 0.11 ($n = 5$) | — |
| SUR2B | 119 ± 19 ($n = 4$) | 1.31 ± 0.12 ($n = 4$) | 52 ± 4[b] ($n = 5$) | 2.60 ± 0.35 ($n = 5$) | 2.21 ± 0.03 ($n = 5$) |

Mean ± S.E.M. values of the ATP concentration producing half-maximal inhibition ($IC_{50}$) or activation ($EC_{50}$) of the channel, of the slope factor (Hill coefficient), and of the maximal current activation (*A*). The number of patches is indicated in parentheses. [a]$IC_{50}$ values are given for Kir6.2/SUR channels, and $IC_{50}$ or $EC_{50}$ values are given for Kir6.2-F333I/SUR channels; [b]activation; [c]inhibition.

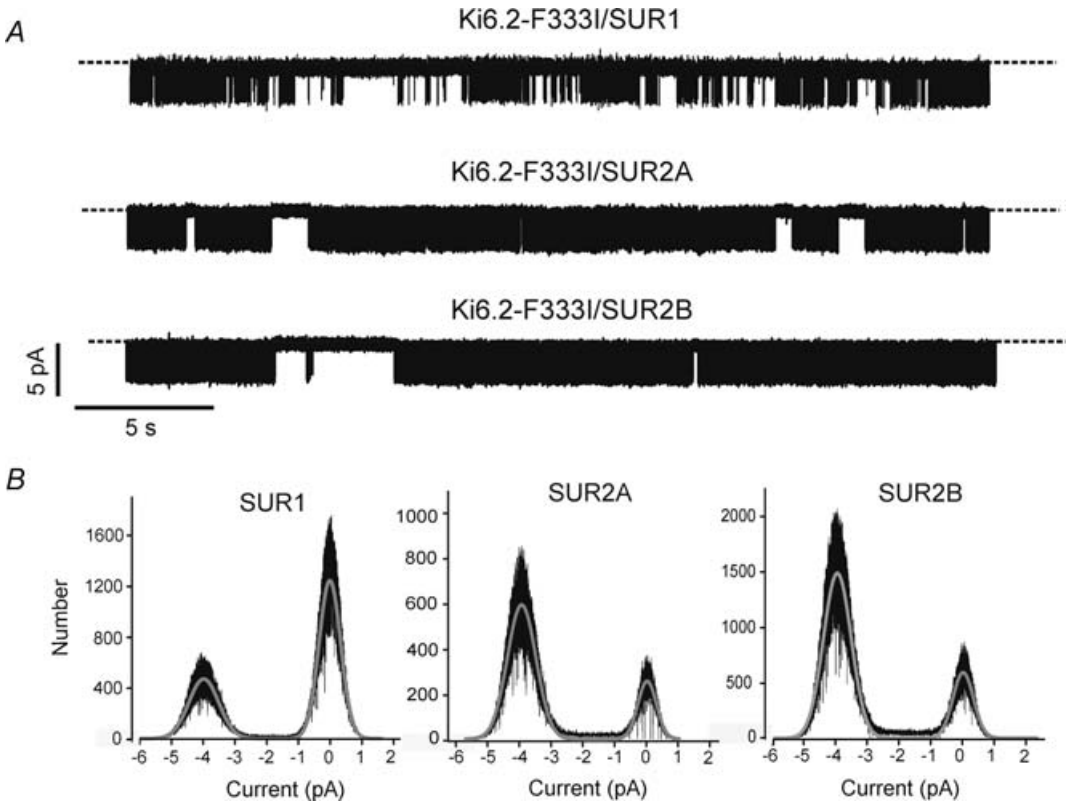


**Figure 2. Unitary currents**
*A*, single-channel currents recorded at −60 mV from mutant $K_{ATP}$ channels, as indicated. *B*, all-point amplitude histograms from the same patches as in *A* for recordings of 30–50 s duration. The continuous lines are fit with the sum of two Gaussian functions. The dashed line indicates the zero current level. Intrinsic open probability ($P_{o(0)}$) was 0.41 (SUR1), 0.70 (SUR2A) and 0.69 (SUR2B).

**Table 2. Single-channel open probability ($P_{o(0)}$) of wild-type and Kir6.2-F333I mutant channels**

| | $P_{o(0)}$ | |
|---|---|---|
| SUR subtype | Kir6.2 | Kir6.2-F333I |
| SUR1 | $0.35 \pm 0.04$ ($n = 7$) | $0.38 \pm 0.08$ ($n = 7$) |
| SUR2A | $0.40 \pm 0.06$ ($n = 6$) | $0.71 \pm 0.04$ ($n = 7$)* |
| SUR2B | $0.41 \pm 0.08$ ($n = 4$) | $0.68 \pm 0.06$ ($n = 6$)* |

Mean ± S.E.M. values of intrinsic open probability ($P_{o(0)}$) measured at −60 mV. *Statistical significance against the wild-type ($P < 0.05$). The number of patches is indicated in parentheses.

provides direct support for the idea that residue F333I may affect the mechanism by which SUR2, but not SUR1, modulates the gating of Kir6.2. It also provides additional evidence that F333I is involved in differential coupling of SUR1 and SUR2 to Kir6.2.

Although we have found no change in gating, the $IC_{50}$ for ATP block of Kir6.2ΔC-F333I channels is 6.4 mM compared with 120 $\mu$M for Kir6.2ΔC (Tammaro *et al.* 2005). This indicates that the F333I mutation must reduce ATP binding/transduction to Kir6.2. However, the ability of SUR to enhance the ATP sensitivity of Kir6.2 seems to be increased by the mutation. Whereas SUR1 and SUR2 enhance the ATP sensitivity of wild-type Kir6.2 (in the absence of $Mg^{2+}$) about ∼10- to ∼20-fold, they potentiate that of Kir6.2-F333I by >30-fold and ∼60-fold, respectively. This suggests that the mutation actually *enhances* the interaction of SUR and Kir6.2 and that further supports the view that this is more effective for SUR2 than SUR1.

### Effects of ATP in the presence of $Mg^{2+}$

A dramatic difference between Kir6.2-F333I/SUR1, Kir6.2-F333I/SUR2A and Kir6.2-F333I/SUR2B channels was observed when ATP was applied in the presence of $Mg^{2+}$ (Fig. 3). MgATP (1 mM) markedly increased Kir6.2-F333I/SUR1 and Kir6.2-F333I/SUR2B currents, but caused a sudden and substantial block of Kir6.2-F333I/SUR2A, followed by a small increase. Current activation was well fit by a single exponential function with a

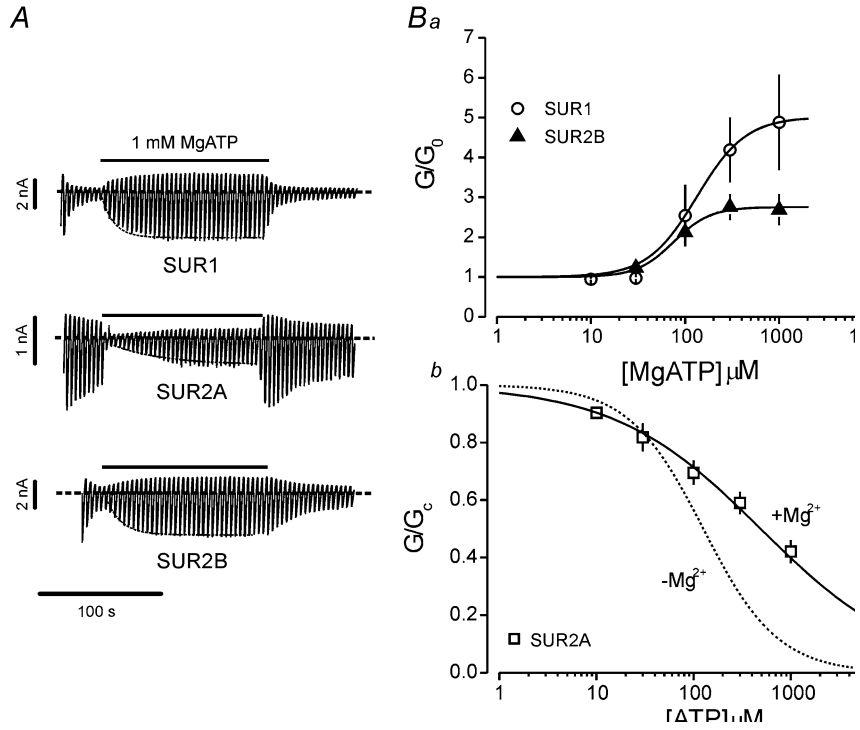


**Figure 3. ATP sensitivity in the presence of $Mg^{2+}$**
*A*, Kir6.2-F333I/SUR1, Kir6.2-F333I/SUR2A and Kir6.2-F333I/SUR2B currents elicited by voltage ramps from −110 to +100 mV. The dotted lines are single exponential fits to the current activation, with time constants of 20 s (SUR1), 49 s (SUR2A) and 28 s (SUR2B). The dashed lines indicate the zero current level. *B*, mean relationship between [MgATP] and $K_{ATP}$ conductance ($G$), expressed relative to the conductance in the absence of nucleotide ($G_0$) for Kir6.2-F333I plus (*a*) SUR1 (○), SUR2B (▲) and (*b*) SUR2A (□). *Ba*, the lines are the best fit of eqn (2) ($EC_{50} = 115$ $\mu$M, $A = 4.6$ for SUR1; $EC_{50} = 53$ $\mu$M, $A = 2.2$ for SUR2B). *Bb*, the smooth line is the best fit of eqn (1) ($IC_{50} = 548$ $\mu$M). The dotted line is the fit to the data in the absence of $Mg^{2+}$ in Fig. 1*B*.

**Table 3. Time constants of Kir6.2-F333I/SUR current activation in response to Mg-nucleotides**

| | Time constant (s) | |
|---|---|---|
| SUR subtype | 1 mM MgATP | 1 mM MgADP |
| SUR1 | $27 \pm 4$ ($n = 10$) | $4.3 \pm 0.3$ ($n = 8$) |
| SUR2A | $75 \pm 14$ ($n = 11$) | $5.9 \pm 1.3$ ($n = 11$) |
| SUR2B | $38 \pm 4$ ($n = 6$) | $6.0 \pm 0.6$ ($n = 7$) |

Data are given as mean ± S.E.M. The number of patches is indicated in parentheses.

time constant of 27 s for Kir6.2-F333I/SUR1, 75 s for Kir6.2-F333I/SUR2A and 38 s for Kir6.2-F333I/SUR2B (Table 3). Thus, the secondary activation of SUR2A channels is not only smaller, but also slower.

Inhibition of Kir6.2-F333I/SUR2A channels by MgATP was ∼fourfold less than that found for ATP in the absence of $Mg^{2+}$ ($IC_{50} = 541\ \mu M$; Fig. 3*Bb*), consistent with the idea that MgATP interaction with the NBDs stimulates channel activity, thereby antagonizing the ATP block at Kir6.2. This effect was very much greater for SUR1- and SUR2B-containing channels: indeed, MgATP now produced channel *activation* rather than block. At 1 mM MgATP, currents increased ∼fivefold for SUR1- and ∼twofold for SUR2B-containing channels, although activation declined at 10 mM MgATP (Fig. 3*Ba*). Fitting the data points up to 1 mM ATP with eqn (2), gave $EC_{50}$ values of 113 and 52 $\mu$M for SUR1 and SUR2B channels, respectively (Table 1).

We have shown elsewhere that the stimulatory effect of MgATP on Kir6.2-F333I/SUR1 channels is abolished by mutations in the NBDs of SUR1 that impair ATP binding and hydrolysis (Tammaro *et al.* 2005). This implies that channel activation is due to MgATP binding/hydrolysis at the NBDs of SUR1 and not to other effects such as, for example, $PIP_2$ generation. Here, we report that these channels were blocked by ATP with an $IC_{50}$ of $681 \pm 76\ \mu M$ ($n = 7$), a value not significantly different from that found for Kir6.2-F333I/SUR2A. This suggests that the different behaviours of SUR1-, SUR2A- and SUR2B-containing channels reflect variations in nucleotide binding/hydrolysis by the NBDs, or in the mechanism by which nucleotide occupancy at the NBDs is translated into opening of the Kir6.2 pore.

## Effects of ADP and MgADP

To discriminate between these possibilities, we tested the ability of MgADP to activate Kir6.2-F333I channels. Figure 4*A* shows that MgADP activates SUR1-, SUR2A- and SUR2B-containing channels to similar extents. Maximal activation and $EC_{50}$ (∼50 $\mu$M) were also similar (Fig. 4*B*, Table 4). This suggests transduction of MgADP binding into channel opening occurs with similar efficacy for all three channel types. Thus the different responses to MgATP must reflect differences in ATP binding/hydrolysis by SUR1, SUR2A and SUR2B.

All three channel types displayed a similar time course of stimulation by 1 mM MgADP, with time constants of 4.3 s for Kir6.2-F333I/SUR1, 5.9 s for Kir6.2-F333I/SUR2A and 6.0 s for Kir6.2-F333I/SUR2B (Table 3). These values are significantly slower than the solution exchange rate ($\tau < 400$ ms for block by 1 mM ATP), and >fourfold faster than the corresponding rates of activation by MgATP. This is consistent with the idea that MgATP must first be hydrolysed to MgADP before channel activation can occur (Zingman *et al.* 2001).

We also assessed the sensitivity of Kir6.2/SUR2A and Kir6.2-F333I/SUR2A to ADP in the absence of $Mg^{2+}$, where nucleotides interact solely with Kir6.2. Figure 4*C* shows ADP blocked mutant channels (at Kir6.2) less than wild-type channels ($IC_{50}$ of $1.7 \pm 0.2$ mM, $n = 4$ *versus* $82 \pm 12\ \mu M$, $n = 6$). This inhibition of Kir6.2/SUR2A is comparable to that found for Kir6.2/SUR1 (64 $\mu$M; Dabrowski *et al.* 2004). Significantly, mutant channels were not blocked by 100 $\mu$M ADP in the absence of $Mg^{2+}$, whereas they showed marked activation in 2 mM $Mg^{2+}$ (Fig. 4*B*). Thus at this MgADP concentration, it is possible to study channel activation (via SUR) in the absence of block (at Kir6.2). It is noteworthy that there is no MgADP concentration at which such a separation is achieved for wild-type channels (Fig. 4*C*).

As we discussed above, the effect of MgADP on wild-type $K_{ATP}$ channels reflects the balance between its inhibitory action at Kir6.2 and its stimulatory action mediated via the NBDs of SUR (Tucker *et al.* 1997). In contrast, GDP has little inhibitory effect at Kir6.2 (Trapp *et al.* 1997; Tammaro *et al.* 2006); thus this nucleotide can be used to study Mg-nucleotide activation of wild-type and Kir6.2-F333I channels in the absence of block at Kir6.2 and to determine if the F333I mutation alters the interaction of Mg-nucleotide diphosphates with the $K_{ATP}$ channel. At a concentration of 100 $\mu$M, MgGDP activated wild-type and mutant channels to similar extents, independent of whether they were composed of SUR1, SUR2A or SUR2B (Fig. 4*D*). This suggests that the F333I mutation does not alter MgGDP binding to the NBDs of SUR, nor the way in which this binding is transduced into channel opening. The marked increase in MgATP activation of SUR1 and SUR2A channels must therefore reflect an increase in MgATP binding/hydrolysis.

## Effects on whole-cell currents

Finally, we asked if the different MgATP sensitivities of channels composed of SUR1, SUR2A or SUR2B led to

**Table 4. ADP sensitivity of Kir6.2-F333I/SUR channels**

| SUR subtype | $EC_{50}$ ($\mu$M) 2 mM $Mg^{2+}$ | Slope factor 2 mM $Mg^{2+}$ | *A* 2 mM $Mg^{2+}$ |
|---|---|---|---|
| SUR1 | $50 \pm 14$ ($n = 5$) | $0.89 \pm 0.21$ ($n = 5$) | $4.2 \pm 0.19$ ($n = 5$) |
| SUR2A | $52 \pm 12$ ($n = 6$) | $0.96 \pm 0.50$ ($n = 6$) | $4.38 \pm 0.53$ ($n = 6$) |
| SUR2B | $42 \pm 16$ ($n = 5$) | $1.23 \pm 0.09$ ($n = 5$) | $4.09 \pm 0.06$ ($n = 5$) |

Mean $\pm$ S.E.M. values of the MgADP concentration producing half-maximal activation ($EC_{50}$) of the channel, of the slope factor (Hill coefficient) and of the maximal current activation (*A*). The number of patches is indicated in parentheses.

variation in the whole-cell $K_{ATP}$ currents. Resting currents were almost undetectable for wild-type Kir6.2/SUR1 and Kir6.2/SUR2A (Tammaro *et al.* 2006), as expected because of the high resting intracellular [ATP] in the oocyte. Metabolic inhibition (3 mM azide) activated Kir6.2/SUR1 but not Kir6.2/SUR2A (Tammaro *et al.* 2006). Kir6.2-F333I channels showed very different resting currents, depending on the SUR isoform: SUR1 currents

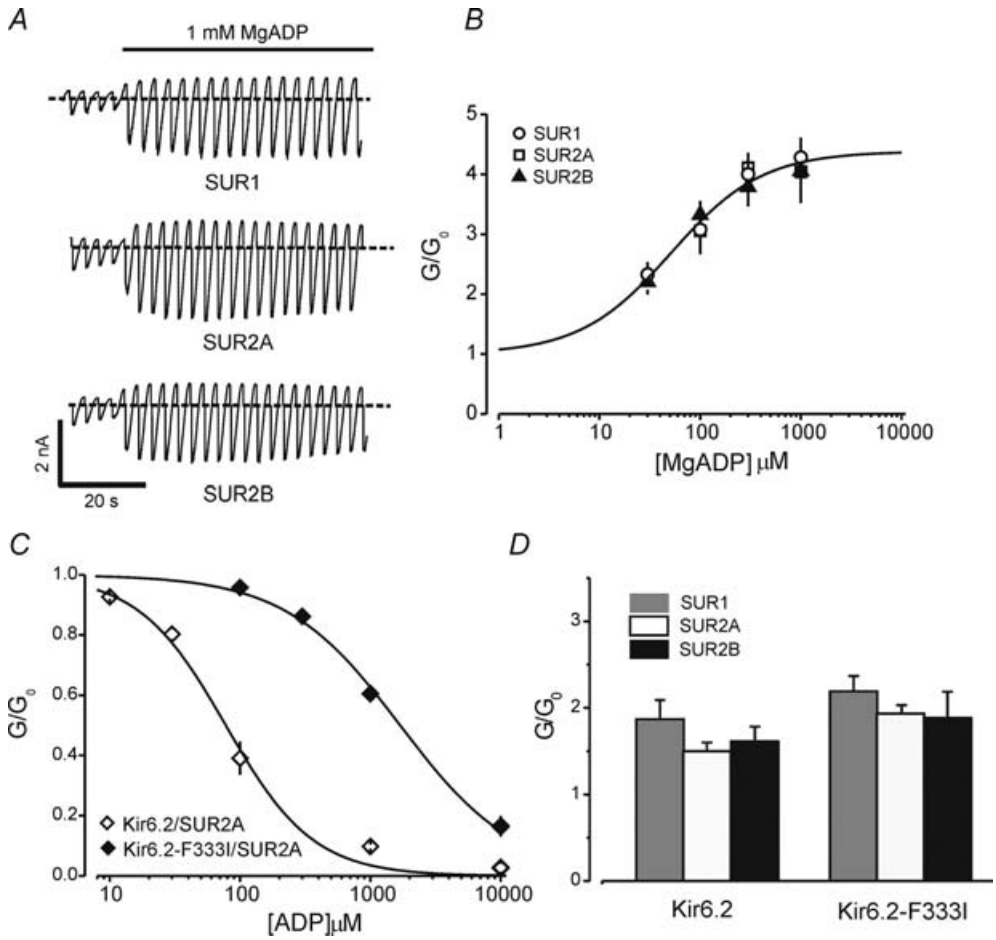


**Figure 4. ADP and GDP sensitivities in the presence and absence of $Mg^{2+}$**
*A*, Kir6.2-F333I/SUR1, Kir6.2-F333I/SUR2A and Kir6.2-F333I/SUR2B currents elicited by voltage ramps from −110 to +100 mV. MgADP (1 mM) was applied as indicated. *B*, mean relationship between [MgADP] and $K_{ATP}$ conductance (*G*), expressed relative to the conductance in the absence of nucleotide ($G_0$) for F333I plus SUR1 (○), SUR2A (□) and SUR2B (▲). The line is the best fit of eqn (2) ($EC_{50} = 52$ $\mu$M, $A = 4.4$). *C*, mean relationship between [ADP] and $K_{ATP}$ conductance (*G*), expressed relative to the conductance in the absence of nucleotide ($G_0$), in $Mg^{2+}$-free solution. Kir6.2/SUR2A (◇, $n = 4$) or Kir6.2-F333I/SUR2A (◆, $n = 6$). The lines are the best fit of eqn (1) ($IC_{50} = 80$ $\mu$M, $h = 1.2$ for Kir6.2/SUR2A; $IC_{50} = 1.7$ mM, $h = 1.0$ for Kir6.2-F333I/SUR2A). *D*, mean conductance recorded in the presence of 100 $\mu$M MgGDP expressed relative to the mean of that recorded in the absence of the nucleotide, for the channels indicated. The number of patches was 4–7 in each case.

were ∼sixfold larger, and SUR2B currents ∼twofold larger, than SUR2A currents (Fig. 5). Metabolic inhibition increased all three types of $K_{ATP}$ current to a similar level (Fig. 5), suggesting that differences in resting current are not due to variable expression, but to differences in metabolic sensitivity. A similar difference in resting current has been observed when other Kir6.2 ND mutations are coexpressed with either SUR1 or SUR2A (Tammaro *et al.* 2006).

## Discussion

Our results demonstrate that the F333I mutation in Kir6.2 reduces the ATP sensitivity of cardiac (Kir6.2/SUR2A) and nonvascular smooth muscle (Kir6.2/SUR2B) types of $K_{ATP}$ channel, as well as $\beta$-cell channels (Kir6.2/SUR1). Consistent with the smaller resting whole-cell currents, mutant channels containing SUR2A were blocked significantly more by MgATP than those containing SUR2B or SUR1. Further analysis suggested that the marked difference in ATP sensitivity between the different channel types is most likely to be due to the ability of the Kir6.2 mutation to increase MgATP binding/hydrolysis at the NBDs of SUR1 and SUR2B, but not SUR2A. In addition, the F333I mutation altered the coupling of Kir6.2 to SUR2, but not SUR1; it thereby increased the open probability of SUR2 channels as well as the ability of SUR2 to enhance the ATP sensitivity of Kir6.2.

### Effects on intrinsic gating

The F333I mutation had no effect on the intrinsic open probability of Kir6.2/SUR1, yet increased that of Kir6.2/SUR2A and Kir6.2/SUR2B. The sulphonylurea receptor has at least two effects on $K_{ATP}$ channel gating, one stimulatory and one inhibitory (Babenko *et al.* 1999*b*). Thus, it is possible that the F333I mutation abolishes the inhibitory action of SUR2A and SUR2B, but not SUR1, on gating: however, this requires confirmation. Modulation of Kir6.2 gating by SUR is thought to be mediated via interaction of the first five transmembrane domains, and the following cytosolic loop, of SUR with the N terminus of Kir6.2. This is because deletions/mutations in the Kir6.2 N terminus produce an increase in $P_{o(0)}$ that manifests only when Kir6.2 is coexpressed with SUR1 (Babenko *et al.* 1999*a*; Koster *et al.* 1999; Reimann *et al.* 1999). Our data provide evidence that SUR1 also interacts with the C terminus of Kir6.2 to modulate gating. Consistent with the functional data, a molecular model of Kir6.2 locates F333 close to the N terminus of Kir6.2 (Antcliff *et al.* 2005). Because the Kir6.2-F333I mutation modulates the $P_{o(0)}$ of $K_{ATP}$ channels containing SUR2A and SUR2B, but not SUR1, our data also provide evidence that the SUR residues with which F333I interacts may vary between SUR1 and SUR2.

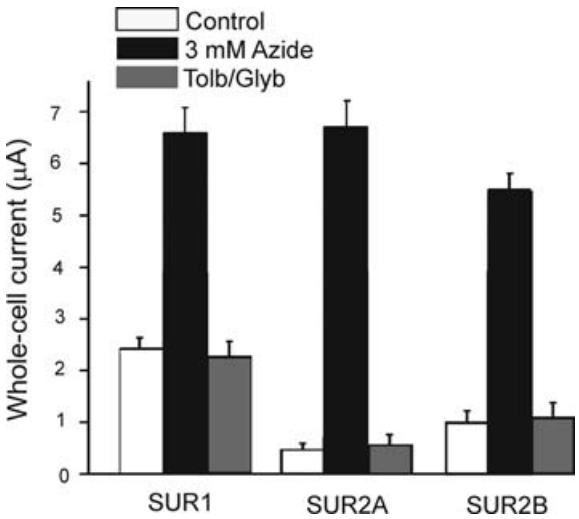


**Figure 5. Metabolic regulation of whole-cell $K_{ATP}$ currents**
Whole-cell currents evoked by voltage steps to −30 mV from a holding potential of −10 mV, before (control) and after application of 3 mM azide, and in the presence of 3 mM azide + 0.5 mM tolbutamide (SUR1) or 10 $\mu$M glibenclamide (SUR2A, SUR2B). The number of oocytes was 4–8 in each case.

### Enhanced ATP sensitivity

Our results suggest that the F333I may influence the ability of SUR to increase the sensitivity of Kir6.2 to ATP. Previous studies have suggested that SUR modifies the binding pocket itself (Dabrowski *et al.* 2004), which is consistent with the location of F333, close to the phosphate tail of ATP. The region of SUR involved is unknown, but evidence suggests that it does not involve the first five transmembrane domains (Chan *et al.* 2003).

### Activation by Mg-nucleotide diphosphates

We compared MgGDP activation of wild-type and mutant $K_{ATP}$ channels containing different SUR isoforms, as GDP concentrations <1 mM produce little block at Kir6.2 (Trapp *et al.* 1997; Tammaro *et al.* 2006). This allows the effects of Mg-nucleotide interaction with the NBDs of SUR to be studied in isolation. Our results confirm earlier findings that MgGDP activates Kir6.2/SUR1 and Kir6.2/SUR2A channels to similar extents (Tammaro *et al.* 2005), and demonstrate that this is also true for Kir6.2/SUR2B channels. Importantly, we found that MgGDP activation was unaffected by the Kir6.2-F333I mutation, for all three types of channel. This is in contrast to the marked stimulation of MgGDP activation produced by ND mutation Kir6.2-R201H (Tammaro *et al.* 2006). Thus Kir6.2 ND mutations may mediate their effects on the ability of SUR to enhance channel activity by different mechanisms.

The marked block by ADP at Kir6.2 has precluded measurements of $IC_{50}$ for MgADP activation of wild-type channels. Use of the F333I mutant allows determination of whether the various SUR differ in their ability to couple MgADP binding at the NBDs to changes in channel activity. As we show, this does not appear to be the case. Kir6.2-F333I channels containing different SUR subunits show a similar time course, $EC_{50,}$ and maximal activation by MgADP. This means that both MgADP binding, and the transduction mechanism that links nucleotide binding to channel opening, do not differ between SUR subtypes. The $EC_{50}$ for MgADP activation ($\sim$50 $\mu$M) is reasonably close to the $K_i$ of 70–100 $\mu$M measured for MgADP displacement of [$^{32}$P]azidoATP photoaffinity labelling to different SUR (Matsuo *et al.* 2000).

### Activation by MgATP

Unlike MgADP, MgATP had different effects on Kir6.2-F333I channels containing SUR1, SUR2B and SUR2A: it blocked SUR2A but activated SUR1 and SUR2B currents. The dramatic activation of Kir6.2-F333I/SUR1 appears to be due to ATP binding/hydrolysis by the NBDs of SUR1, as it is prevented by mutations that impair ATP binding/hydrolysis and is not produced by non-hydrolysable ATP analogues (Tammaro *et al.* 2005). Our results suggest that ATP binding/hydrolysis is less for Kir6.2-F333I/SUR2B and very substantially lower for Kir6.2-F333I/SUR2A. However, the shift in the ATP concentration-inhibition curve produced by $Mg^{2+}$ argues that MgATP does produce some activation of Kir6.2-F333I/SUR2A (approximately equal to that observed when the Walker A lysines in NBDs of SUR1 are mutated (Gribble *et al.* 1997*b*).

Given the apparent lack of effect on MgADP binding/transduction, it seems likely that the F333I mutation affects ATP hydrolysis rather than ATP binding at SUR. The differential effect of the mutation in SUR1- and SUR2-containing channels may therefore be related to the ability of NBD2 of SUR1 and SUR2B to hydrolyse MgATP more effectively than that of SUR2A (Masia *et al.* 2005). If ATP hydrolysis by SUR2A is already very low, then even if it is stimulated by the F333I mutation to the same extent as that of SUR2B, the effect may be too small to produce the same degree of channel activation. Alternatively, the F333I mutation may potentiate ATP hydrolysis by SUR1 and SUR2B, but not SUR2A. The possibility that Kir6.2 can modulate ATP hydrolysis at the NBDs of SUR is not unreasonable. First, microscopic reversibility predicts that if ATP hydrolysis can modulate the function of Kir6.2, then the reverse must also be true. Second, the ATPase activity of the purified $K_{ATP}$ channel complex (Mikhailov *et al.* 2005) is significantly higher than that of either SUR1 (De Wet *et al.* 2005) or the isolated NBDs of SUR1 (Masia *et al.* 2005). Our results suggest that Kir6.2 may be involved in the mechanism by which this modulation occurs. More specifically, they suggest the C terminus of Kir6.2 may be involved.

### Metabolic sensitivity

It is striking that whereas Kir6.2/SUR1 channels are potently activated by metabolic inhibition, Kir6.2/SUR2A channels are not (Tammaro *et al.* 2006). In contrast, Kir6.2-F333I/SUR2A channels exhibit a small resting current and are further increased by azide. Although there was no obvious difference in azide-activated currents when Kir6.2-F333I was coexpressed with SUR1, SUR2A or SUR2B, the resting currents (in control solution) were sixfold larger for SUR1, and twofold larger for SUR2B, than SUR2A channels. Since activation by MgADP is similar for all three types of channel, and inhibition by ATP (in the absence of $Mg^{2+}$) is identical for SUR2A and SUR2B channels, these results suggest that the difference in the resting whole-cell currents reflects the differences observed in MgATP activation. Thus the rate of MgATP hydrolysis at the NBDs of SUR appears to influence $K_{ATP}$ channel activity in the intact cell. This is in agreement with the relative concentrations of MgATP and MgADP in cells and suggests that the NBDs will preferentially bind MgATP, and that they will only be occupied by MgADP following binding and hydrolysis of MgATP (Tarasov *et al.* 2004).

### Physiological relevance

We have previously suggested that the lack of obvious cardiac effects in patients with the R201H and Q52R ND mutations is due to marked to differences in MgATP handling by SUR1 and SUR2A (Tammaro *et al.* 2006). A similar argument can been made for the F333I mutation, as our results reveal a much greater reduction in MgATP sensitivity, and increase in whole-cell $K_{ATP}$ currents, for Kir6.2-F333I/SUR1 than Kir6.2-F333I/SUR2A. However, this needs to be confirmed for the heterozygous state. In contrast to SUR2A channels, we found that the MgATP sensitivity of SUR2B channels was significantly shifted by the F333I mutation. This raises the possibility that that nonvascular smooth muscles and neurons expressing Kir6.2/SUR2B channels (Liss *et al.* 1999) may be affected in patients carrying ND mutations. This may perhaps contribute to the extra-pancreatic symptoms characteristic of patients with DEND syndrome (Hattersley & Ashcroft, 2005).

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