Molecular mechanisms of congenital hyperinsulinism due to autosomal dominant mutations in ABCC8

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Abstract
Congenital Hyperinsulinism (CHI) is a rare heterogeneous disease characterized by unregulated insulin secretion. Dominant mutations in ABCC8 causing medically unresponsive CHI have been reported; however, the molecular mechanisms are not clear. The molecular basis of medically unresponsive CHI due to dominant ABCC8 mutations has been studied in 10 patients, who were medically unresponsive to diazoxide (DZX), and nine of whom required a near-total pancreatectomy, and one partial pancreatectomy. DNA sequencing revealed seven dominant inactivating heterozygous missense mutations in ABCC8, including one novel and six previously reported but uncharacterized mutations. Two groups of mutations with different cellular mechanisms were characterized. Mutations in the transmembrane domain (TMD) were more responsive to channel activators such as DZX, MgADP and metabolic inhibition. The trafficking analysis has shown that nucleotide-binding domain two (NBD2) mutations are not retained in the endoplasmic reticulum (ER) and are present on the membrane. However, the TMD mutations were retained in the ER. D1506E was the most severe SUR1-NBD2 mutation. Homologous expression of D1506E revealed a near absence of KATP currents in the presence of DZX and intracellular MgADP. Heterozygous expression of D1506E showed a strong dominant-negative effect on SUR1-Kir6.2 currents. Overall, we define two groups of mutation with different cellular mechanisms. In the first group, channel complexes with mutations in NBD2 of SUR1 traffic normally but are unable to be activated by MgADP. In the second group, channels mutations in the TMD of SUR1 are retained in the ER and have variable functional impairment.

Introduction
Congenital hyperinsulinism (CHI) is a rare disease characterized by inappropriate insulin secretion leading to severe hypoglycaemia with potential consequences that can lead to neurological damage (1,2).

There are two major histological subtypes of CHI: focal and diffuse. Diffuse disease affects the entire pancreas (all the pancreatic β-cells) (3) and patients may need to undergo a near-total pancreatectomy. In most cases, diffuse CHI is inherited in an autosomal recessive manner whereas the focal form is sporadic (4).

CHI is caused by mutations in nine known genes (ABCC8, KCNJ11, GLUD1, GCK, HADH, HNF4A, HNF1A, SLC16A1 and UCP2). The vast majority of mutations can be attributed to the former two genes. The ABCC8 and KCNJ11 genes encode for the proteins SUR1 and Kir6.2, respectively (5). These proteins co-assemble into
a single ATP-sensitive potassium channel (K<sub>ATP</sub>), which plays a vital role in regulating insulin secretion from ß-cells. The oral administration of diazoxide (DZX), a K<sub>ATP</sub> channel opener, is the mainstay of medical treatment in CHI. It is known that DZX inhibits the influx of Ca<sup>2+</sup> and prevents the release of insulin, irrespective of the blood glucose levels (6,7). This drug however is only effective in patients who have some functional K<sub>ATP</sub> channels, which is generally a feature of the less common dominant form of CHI. Diazoxide, however, is ineffective in patients with the recessive form of CHI.

Mutations in ABCC8 and KCNJ11 can be classified under two categories based on their functional impediment. Class 1 mutations generate proteins which fail to reach the surface of the plasma membrane, and can be due to the impaired synthesis or maturation of the SUR1 protein in the ER or Golgi apparatus. Class 2 mutations, however, produce K<sub>ATP</sub> channel complexes which are embedded in the cell membrane but are non-functional. The sensitivity to nucleotides such as MgADP is significantly blunted in these defective K<sub>ATP</sub> channels, hence they remain closed even when the glucose levels are low (8,9).

The two nucleotide-binding domains (NBD) of the SUR1 protein are responsible for sensing the changes in nucleotide levels inside ß-cells. It has been postulated that ATP hydrolysis is achieved through conformational changes occurring at the NBD’s of SUR1 (10). The SUR1 NBD also contains two conserved sequences known as the Walker A and Walker B motifs (11). The close proximity of the NBD’s in the SUR1 subunit permits the formation of two nucleotide-binding sites, which hydrolyse MgATP. However, it has shown that NBD1 has a higher affinity for MgATP with decreased rate of hydrolysis, and it is hypothesized that MgADP is needed for the activation of the K<sub>ATP</sub> channel through nucleotide-binding domain two (NBD2) (12). Hence, SUR1 is the regulatory subunit which provides the channel its sensitivity to Mg<sup>2+</sup> nucleotides, and to both types of drugs including the inhibiting sulfonylureas and activating DZX.

Autosomal dominant inheritance of CHI is uncommon, with patients having mild symptoms and they are generally responsive to medical therapy. This has led to the hypothesis that dominant mutations yield K<sub>ATP</sub> channels which are responsive to medical therapies, hence patients do not require a pancreatectomy (13,14). Previously all in-vitro studies have demonstrated that dominant mutations in CHI involve amino acid changes which allow normal trafficking of mature K<sub>ATP</sub> channels to the cell surface (13).

However, there are now reported cases of autosomal dominant mutations which cause diffuse CHI and which are unresponsive to medical therapy. These are indistinguishable from the common diffuse form of CHI due to recessive K<sub>ATP</sub> channel mutations (15,16). MacMullen and colleagues described patients with dominant ABCC8 mutations causing medically unresponsive CHI, where almost all the patients required a near-total pancreatectomy (15). The functional analyses showed a severe impairment in the channels response to agonists; however, the channels were able to traffic to the membrane as has been described for all dominant DZX responsive mutations. Paternal mutations causing diffuse disease have also been described (17-22). It has been largely accepted that paternal mutations can cause focal, diffuse and atypical CHI.

The aims of this study were to describe the phenotype of a cohort of patients with medically unresponsive CHI due to dominant ABCC8 gene mutations, and to gain a mechanistic insight into how these mutations lead to CHI.

### Results

#### Clinical characteristics and histology

The key clinical features of all probands have been presented in Table 1, and have also been described in our previous reports (16,21). Proband 6 however, carries a novel mutation (I1425L). He was born at 36+3 weeks gestation at a birth weight of 4310 g, and required a pancreatectomy at 10 weeks to control the hypoglycaemia. Histological examination confirmed diffuse disease. Post-operatively, the proband was continued on octreotide, plus

<table>
<thead>
<tr>
<th>Proband</th>
<th>BW (g)</th>
<th>Gestation</th>
<th>Age of presentation</th>
<th>Diazoxide responsive</th>
<th>Pancreatectomy</th>
<th>Mutation</th>
<th>ABCC8 exon</th>
<th>Region</th>
<th>Inheritance</th>
<th>Histology</th>
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<tbody>
<tr>
<td>1</td>
<td>3190</td>
<td>33</td>
<td>Day 1</td>
<td>No</td>
<td>8 weeks</td>
<td>D1506E</td>
<td>(c.4518C&gt;A)</td>
<td>37</td>
<td>Maternal</td>
<td>Diffuse</td>
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<tr>
<td>2</td>
<td>4528</td>
<td>39</td>
<td>Day 3</td>
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<td>5 weeks</td>
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<td>36+2</td>
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<td>8 weeks</td>
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<td>Diffuse</td>
</tr>
<tr>
<td>6</td>
<td>4310</td>
<td>36+3</td>
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<td>(c.2573A&gt;C)</td>
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<td>7</td>
<td>4140</td>
<td>39</td>
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<td>Yes</td>
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<td>7 months—Pancreatectomies Yes</td>
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<td>TMD0</td>
<td>Diffuse</td>
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<td>37</td>
<td>Day 1</td>
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<td>Partial at 4.5 years</td>
<td>A1263T</td>
<td>(c.3787&gt;A)</td>
<td>31</td>
<td>TMD2</td>
<td>Diffuse</td>
</tr>
<tr>
<td>10</td>
<td>2700</td>
<td>42</td>
<td>2½ years</td>
<td>No</td>
<td></td>
<td>A1263T</td>
<td>(c.3787&gt;A)</td>
<td>31</td>
<td>TMD2</td>
<td>Not done</td>
</tr>
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</table>
4 hourly bolus feeds, with Vitajul (glucose powder) and Creon (pancreatic enzyme supplements). Genetic tests on the parents showed that the mutation had arisen de novo in proband 6. The pedigrees are shown in Figure 1. Microsatellite analysis of chromosome 11p15 in probands 3 and 5 revealed no loss of heterozygosity. Pancreatic histological specimens for the remaining probands were not available for the same analysis.

Genetics

The patients in this study have autosomal dominant ABCC8 mutations which are unresponsive to DZX, the mechanism of this is largely unknown (Table 1). A single patient has a novel SUR1 mutation (I1425L), whilst the others have previously reported dominantly inactivating heterozygous missense mutations in ABCC8 (16,21). It is worth noting that the mutations in SUR1 can be grouped into two kinds of mutations: those located in NBD2 and those in the transmembrane domains (TMDs) (Fig. 2). The dominant mode of transmission can be confirmed for D1506E mutation which has been seen in two families as shown in this cohort (16). This mutation has also been seen in another proband where it was first reported by other researchers (20). A113V is a paternally inherited mutation which has also been observed by Otonkoski and colleagues, although for the G92D mutation they do not state if the mutation was paternally inherited or whether it was de novo (23).

Determining the functional impact of SUR1 mutants using $^{86}$Rb$^+$ efflux

To ascertain the functional impact of the SUR1 mutations, we used $^{86}$Rb$^+$ efflux studies as an initial screen. As expected, WT K$_{ATP}$ channels were strongly activated by 100 µM DZX producing a flux of 28.1 ± 2.7% (Fig. 3A and Table 2). In comparison, D1506E (NBD mutant) and G92D (TMD mutant) were unresponsive to DZX (Fig. 3A). All the mutants displayed between 3.6% and 12.2% activity under DZX in comparison with the WT which is very similar to baseline efflux values (Table 2). In Figure 3B, it can be seen that metabolic inhibition (MI) however did induce an effect on the G92D mutation. The A113V mutation has also shown activation under MI (Table 2). The data derived from the G92D mutant suggest that there are some functional K$_{ATP}$ channels which are able to respond to MI but not to DZX. In cells expressing mutant K$_{ATP}$ channels, it suggests that they are unable to respond to the increasing concentrations of MgADP which occurs as a result of adding metabolic inhibitors like NaCN and 2-Deoxy-D-Glucose. The SUR1 subunit confers the channels response to MgADP in the K$_{ATP}$ channel complex (12), and this has clearly been impaired.

Whole-cell patch-clamp analysis of the currents produced by homozygous expression of WT and mutant K$_{ATP}$ channels in HEK293 cells

We next examined the behaviour of the WT and mutant channels using whole-cell patch-clamp. These studies from whole-cell recordings confirmed that mutations in NBD2 led to severely blunted responses to DZX compared with WT (Fig. 4 and Table 2). The D1506E mutant shows no response to DZX, and in comparison with the WT K$_{ATP}$ channels it does not produce any outward current between the studied range of $-100$ mV to $-60$ mV (Fig. 4B). This mutation is located within the Walker B motif of NBD2, which is a highly conserved region. The M1514K and G1485E mutations are also located within the NBD2 and they have also shown significantly lower responses to DZX than WT channels, with only a tiny current of $0.65 ± 2.6$ pA/pF and $-5.79 ± 5.8$ pA/pF, respectively. This is consistent with the data obtained from the $^{86}$Rb$^+$ flux assay. In the group of SUR1 TMD mutants, the A113V, A1153V and A1263T mutants were also not responsive to

![Figure 1. Pedigrees of 10 patients with autosomal dominant hyperinsulinism associated with K$_{ATP}$ channel mutations. Males are depicted as squares, females as circles. Nc, not carrier of mutation, Mc, mutation carrier, Un, unaffected.](image)
DZX (Fig. 4 and Table 2); however, within the same intracellular nucleotide conditions of low ATP (0.1 mM), the G92D mutant produced a response to DZX generating a current of 55 ± 35.2% (Fig. 4D and Table 2).

We next studied the response of representative mutants in single channel inside-out patches to nucleotides (Fig. 4A1–C1). WT channels were maximally inhibited with 1 mM ATP after patch excision to determine the amount of current in the patch. 1 mM ADP was applied (in the presence of 0.1 mM ATP) to activate currents. WT channels were activated to 83.6 ± 6.6% of their maximum. In comparison, the NBD2 mutant, D1506E did not respond to MgADP producing a significantly low current of 4.5 ± 1.8% of its maximum. In contrast, the TMD mutant, A113V did show a response comparable with WT KATP channels generating a current of 83.5 ± 7.3% of its maximum (Fig. 4E and Table 2).

Effect of different intracellular nucleotide concentrations on mutant $K_{ATP}$ channel activity

The I1425L (NBD2) mutant was not responsive to DZX in the 86Rb+ flux assay or to MI producing efflux values of only 3.7 ± 0.1% and 6.9 ± 0.2%, respectively (Table 2). However, the I1425L KATP channel did activate with DZX in the whole-cell patch-clamp experiment by generating a current of 19.73 ± 3.6 pA/pF ($P < 0.01$, Fig. 5). Mutant K$_{ATP}$ channel activation was also observed for the G92D (TMD0) mutant which produced a stronger current of 81.4 ± 35.3 pA/pF. At 0.1 mM ATP the I1425L mutant has an average reversal potential of $-73.2 ± 2.2$ mV. This is very close to $E_K$, and this suggests that the mutant channel is able to generate a KATP channel current. The G92D mutation has an average reversal potential of $-81.4 ± 3.9$ mV, which strongly indicates that the currents produced by the cell are largely conducted by K$^+$ ions. Therefore, G92D is producing a K$_{ATP}$ channel current which is
activated by DZX and inhibited by GLB. It was hypothesized that this activation was due to the low, un-physiological levels of ATP (0.1 mM) in the intracellular pipette solution. To test this theory a further experiment was conducted using 3 mM ATP. Increasing the ATP concentration abolished the activation by DZX in the whole-cell configuration, bringing the average current down to 1.56 ± 2.3 pA/pF for I1425L and 1.9 ± 3.2 pA/pF for G92D (Fig. 5C and D).

### Table 2. Summary of all findings

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Confocal microscopy</th>
<th>$^{86}$Rb$^+$ flux assay</th>
<th>Whole cell patch clamp</th>
<th>Inside-out patch clamp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Co-localization to ER</td>
<td>% DZX activation</td>
<td>% DZX activation</td>
<td>% DZX activation</td>
</tr>
<tr>
<td>WT</td>
<td>33 ± 3 (n = 18)</td>
<td>28.1 ± 2.7</td>
<td>71.1 ± 2.9</td>
<td>35.98 ± 8.6 (n = 9)</td>
</tr>
<tr>
<td>D1506E</td>
<td>30 ± 3 (n = 14)</td>
<td>4.7 ± 0.5</td>
<td>9.1 ± 0.7</td>
<td>−2.88 ± 1.0 (n = 5)</td>
</tr>
<tr>
<td>M1514K</td>
<td>27 ± 4 (n = 15)</td>
<td>4.8 ± 0.2</td>
<td>9.4 ± 0.3</td>
<td>0.65 ± 2.6 (n = 5)</td>
</tr>
<tr>
<td>G1485E</td>
<td>31 ± 3 (n = 11)</td>
<td>3.6 ± 0.2</td>
<td>7.4 ± 0.5</td>
<td>−5.79 ± 5.8 (n = 7)</td>
</tr>
<tr>
<td>I1425L</td>
<td>44 ± 3 (n = 16)</td>
<td>3.7 ± 0.1</td>
<td>6.9 ± 0.2</td>
<td>12.42 ± 4.2 (n = 5)</td>
</tr>
<tr>
<td>G92D</td>
<td>57 ± 4 (n = 19)</td>
<td>9.5 ± 1.0</td>
<td>47.2 ± 2.2</td>
<td>55.0 ± 35.2 (n = 5)</td>
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<tr>
<td>A113V</td>
<td>52 ± 5 (n = 14)</td>
<td>12.2 ± 1.5</td>
<td>37.5 ± 4.8</td>
<td>−1.88 ± 0.9 (n = 7)</td>
</tr>
<tr>
<td>A1263T</td>
<td>67 ± 5 (n = 15)</td>
<td>6.3 ± 0.2</td>
<td>16.9 ± 0.2</td>
<td>−2.48 ± 1.8 (n = 5)</td>
</tr>
</tbody>
</table>

WT and mutant cDNA constructs were individually transfected into HEK293 cells for a series of functional studies. Confocal microscopy was used to determine the subcellular location of mutant KATP channels, by co-transfecting with pDs-Red2-ER and Kir6.2-GFP. The data presented are the mean, sSEM. $^{86}$Rb$^+$ was used as a surrogate to measure the efflux of K$^+$ under stimulated conditions in intact cells. The table shows the % mean $^{86}$Rb$^+$ efflux, ±SEM. Electrophysiological techniques were used to measure the whole-cell and single channel currents. The data show the mean value, ±SEM. DN: Dominant-negative. DZX is 100 µM diazoxide. MI is metabolic inhibition with 2.5 mM NaCN and 20 mM 2-deoxy-D-glucose. (n) Is the number of individual cells used in the experiment.

Figure 4. The effect of channel activators on the activity of WT (SUR1/Kir6.2) and mutant (D1506E/A113V) KATP channels transiently expressed in HEK293 cells. Cells were voltage-clamped and currents were measured in the whole-cell configuration. Cells were held at a holding potential of −80 mV, and subsequently ramped from −150 mV to 50 mV over 1 s (0.2 mV/ms) and then stepped back to −80 mV. Cells were superfused with 5 mM K$^+$ bath solution (CNT), followed by 100 µM DZX (DZX) to activate K$_{ATP}$ currents and 100 µM DZX and 10 µM GLB (DZX+GLB) to inhibit K$_{ATP}$ currents. Representative traces of (A) WT SUR1/Kir6.2 KATP channel, (B) SUR1 (D1506E)/Kir6.2 KATP and (C) SUR1 (A113V)/Kir6.2 KATP channel, showing the current amplitude between −100 mV and −60 mV. Inside-out patch-clamp recordings from HEK293 cells transiently transfected with WT and mutant KATP channels. Recordings were made at −50 mV in symmetrical solutions (140 mM K$^+$/140 mM K$^+$). Currents were blocked using 1 mM ATP, and then 1 mM ADP was used to stimulate currents in the presence of 0.1 mM ATP. Representative inside-out recordings of (A1) WT SUR1/Kir6.2, (B1) SUR1 (D1506E)/Kir6.2 and (C1) SUR1 (A113V)/Kir6.2 channel in the presence of various concentrations of ATP and 1 mM ADP. (D) Graph showing GLB-sensitive current in cells expressing WT SUR1/Kir6.2 and mutant K$_{ATP}$ channels. These data were derived from whole-cell recordings, by subtracting the current that was sensitive to 10 µM GLB, in the presence of 100 µM DZX. The data were analysed using an unpaired t-test. Data are presented as mean ± SEM. (E) Mean percentage of KATP current in 0.1 mM ATP and 1 mM ADP normalized to the overall 1 mM ATP-sensitive current. Data were analysed using a t-test, ***P < 0.001. WT n = 18, D1506E n = 7, A113V n = 8. Data are presented as mean ± SEM.
Dominant-negative effects of SUR1 mutants

To demonstrate a proof of principle, we selected only three mutants, which include two NBD2 mutations (D1506E and M1514K) and one TMD mutant (A113V), and examined whether these mutants acted in a dominant fashion when coexpressed with wild-type channel subunits. To do this, we used both $86^{\text{Rb}}$+ flux and whole-cell patch-clamp. In both of these assays, all the mutants tested impaired the DZX-activated currents but the degree of the effect varied between mutants. However, in all cases it reduced $86^{\text{Rb}}$+ flux and current by over 50% (Fig. 6). The WT KATP channel is clearly stimulated by 100 µM DZX, although these channels appear more responsive to lowering concentrations of ATP (0.1 mM) in the intracellular solution (ICS). This is because the baseline control values are already at around −10 pA/pF even before the addition of DZX, and the actual response to DZX stimulation is variable between the cells. Hence, there is no statistically significant difference between WT control and DZX. However, when the DZX-activated state is compared with DZX- and GLB-inhibited state then there is clearly a statistical significant difference, $P < 0.05$ (Fig. 6).

The D1506E mutant expressed with WT SUR1 does indeed produce a dominant-negative effect, as there is no stimulation with 100 µM DZX. The M1514K is also a NBD mutant but strikingly it retains a partial response to DZX, suggesting different interactions with WT subunits. The data so far suggest that the A113V mutant does not have a major dominant-negative effect on WT SUR1 subunits. The mixed channels are activated with 100 µM DZX, and as described earlier for the WT channels there is also a significant difference between the activated state with DZX and inhibited state with DZX+GLB ($P < 0.05$). The average reversal potential is 70.4 ± 3.0 mV for A113V which is very similar to the WT
channels where the reversal potential is closer to $E_K$ at $-76.13 \pm 1.8$ mV. At this potential, it is clear that the WT and A113V mutant are producing a KATP-specific current.

**Determining the cellular localization of $K_a6.2$ when expressed with SUR1 mutants**

We next examined the cellular distribution of $K_a6.2$ tagged to the green fluorescent protein ($K_a6.2$–GFP) with and without SUR1 and its degree of colocalization with an ER marker (pDsRed2-ER). In the absence of SUR1 coexpression, $K_a6.2$-GFP was largely resident in the ER whilst with SUR1 it translocated away from the ER to the periphery of the cell consistent with the plasma membrane (Fig. 7). $K_a6.2$-GFP+pDsRed2-ER shows that 62 ± 3% of the $K_a6.2$-GFP protein is retained in the ER in the absence of SUR1, but with the addition of WT SUR1 only ∼29% of the retained channels migrate from the ER. When $K_a6.2$-GFP was coexpressed with the SUR1-NBD2 mutants there was comparable co-localization with the ER marker as for WT SUR1 and showed similar distribution to the plasma membrane. We show a representative example (D1506E) in Figure 7. In contrast in the presence of the TMD mutant, A113V $K_a6.2$-GFP was retained in the ER (Fig. 7). The TMD mutants (G92D, A113V and A1263T) also appear to result in increased retention of $K_a6.2$-GFP in the ER in comparison to the NBD mutants (D1506E, M1514K, G1485E and I1425L), $P < 0.001$ (Table 2).

**Figure 6.** Examining the dominant-negative (DN) effect of SUR1 mutations on KATP channel function. Using the $\text{^{86}Rb}^+$ efflux assay to determine the DN effect of SUR1 mutations (D1506E/M1514K/A113V) in HEK293 cells, by transiently expressing $\log$ SUR1 and 500 ng $K_a6.2$. The cells were exposed to five different drug conditions for 5 min, except MI which lasted 15 min. (A) The graph shows the % mean $\text{^{86}Rb}^+$ efflux for cells treated with; DMSO to determine the baseline efflux, 100 μM DZX to activate channels and 100 μM diazoxide and 10 μM glibenclamide to inhibit channels (DZX+GLB). (A1) The graph shows the % mean $\text{^{86}Rb}^+$ efflux for cells treated with; 2.5 mM NaCN and 20 mM 2-Deoxy-D-Glucose (MI) to activate the channels and MI plus 10 μM glibenclamide (MI+GLB) to inhibit the channels. The effect of MI was also tested on un-transfected (UTF) cells and cells expressing WT $K_a6.2$ only. The $\text{^{86}Rb}^+$ efflux was measured as a percentage of the total $\text{^{86}Rb}^+$ in the medium. Data were analysed using one-way ANOVA, followed by Bonferroni post-t-test, $n = 3$ (in triplicates), ***$P < 0.001$. The whole-cell patch-clamp configuration was used on cells expressing WT/mutant $K_a6.2$ channels. Cells were held at a holding potential of −80 mV, and subsequently ramped from −150 mV to 0 mV over 1 s (0.2 mV/ms) and then stepped back to −80 mV. The three conditions used were: control cell perfused with 5 K+ bath solution (CNT), cell perfused with 100 μM DZX, cell perfused with 100 μM DZX and 10 μM GLB. (B) Representative traces showing the current amplitude between −100 mV and −60 mV of WT SUR1/Ka6.2, SUR1 (D1506E)/Ka6.2 and SUR1 (A113V)/Ka6.2, respectively. (C) Graphs showing the mean data at −60 mV from cells transfected with WT SUR1/Ka6.2, SUR1 (D1506E)/Ka6.2 and SUR1 (A113V)/Ka6.2 respectively. Data were analysed using repeated measures ANOVA, followed by Bonferroni post-hoc test, DN WT $n = 7$, DN D1506E $n = 7$ and DN A113V $n = 9$. *$P < 0.05$. **$P < 0.01$. ***$P < 0.001$. **$P < 0.001$. ***$P < 0.001$.
This study has focussed on the functional characteristics of one novel, and six previously reported dominant inactivating mutations in the \textit{ABCC8} gene. There are a total of 10 patients in this study, all of whom were unresponsive to DZX and nine requiring a near-total pancreatectomy, and one a partial pancreatectomy. All the mutations in this study are heterozygous missense mutations. In some cases, the mutation has been inherited in an autosomal dominant manner in all other cases the mutation had arisen \textit{de novo}. Our results demonstrate a clear functional distinction between SUR1 NBD2 and TMD mutants which will now be discussed in greater detail.

**SUR1-NBD2 mutants**

The D1506E mutant was unresponsive to any of the drugs used in this study. This is in agreement with previously published work where higher concentrations of DZX were used (9). The equivalent mutation in the NBD2 of Cystic fibrosis transmembrane regulator (CFTR) has been shown to affect channel activity by possibly affecting the transduction of nucleotide binding or hydrolysis to channel opening and closing, or by affecting the binding or hydrolysis of ATP (24–26). It has been hypothesized that the mutations affect the activity of DZX by altering the MgATP hydrolysis rates, or the mutations change the coupling of the hydrolysis to channel activation (9). It is thought that MgATP hydrolysis somehow causes desensitization to the inhibitory effect of ATP, and so DZX and MgADP enhance the ‘desensitized’ state of the \( K_{\text{ATP}} \) channel (9). Interestingly in our study, the co-expression of the D1506E mutation with WT SUR1 had a pronounced dominant-negative effect on the WT \( K_{\text{ATP}} \) channel subunits suggesting that even a single mutant subunit is enough to impair channel activation.

A paternally inherited mutation A1458T (NBD2) has been previously described (15). This mutation has been studied by inside-out patch-clamp and has shown only 1.06 ± 0.37% response to DZX stimulation in the presence of 0.1 mM ATP, similarly its response to activation by MgADP was only 0.52 ± 0.21% (15). This is in agreement with the NBD2 mutations particularly D1506E described in this study.

Overall, our data show that, in comparison with WT, all the mutants are relatively insensitive to DZX. This is more pronounced for all the NBD2 mutants that show very little activation, whereas the TMD mutants are less insensitive.

**Differences between the TMD mutants**

In this study, there are three paternal mutations: G92D, A113V and A1263T. The former two mutants have been identified in a different series of patients (23), and the A1263T mutation is present in two probands in this study. For A113V and A1263T, the inheritance has always been paternal. The difference between the mutants is that the SUR1 TMD0 mutants (G92D and A113V) produce significantly higher responses to DZX and MI compared with A1263T. The A1263T TMD2 mutant, however, is unresponsive to DZX, both in the \( ^{86}\text{Rb}^{+} \) flux assay and the whole-cell patch-clamp experiments. The DZX unresponsiveness of the A1263T mutant fits well with the severe clinical phenotype of the patient. It appears that this mutant is more severe than the TMD0 mutants. Interestingly the trafficking correction drugs that are currently being tested only appear to work on mutants with the TMD0 of SUR1 (27).

**Mutants displaying DZX responsiveness in vitro**

The SUR1 TMD0 mutant G92D and NBD2 mutant I1425L showed a response to DZX which does not correlate with the observed clinical phenotype of DZX unresponsive disease. The patients who have these mutations were unresponsive to 20 mg/kg/day of DZX which is the maximum dose recommended for the drug. G92D was relatively unresponsive in the \( ^{86}\text{Rb}^{+} \) flux assays but a response was observed using whole-cell patch-clamp recordings when the intracellular pipette solution contained 0.1 mM ATP. We hypothesized that the intracellular ATP concentration might be affecting the response, so we studied this mutation using more physiological ATP concentrations (3 mM). In this
case the mutant, G92D was unresponsive to DZX in contrast to WT K\textsubscript{ATP}. I1425L also showed some but significantly reduced DZX sensitivity in the standard whole-cell conditions but little DZX-induced Rb\textsuperscript{65} flux suggesting similar considerations pertain.

The novel mutant I1425L (NBD2) provides an interesting paradigm of how changes in amino acid can lead to changes in disease manifestation. The isoleucine amino acid on the position 1425, has been studied by Babenko and colleagues (28). They describe a patient with permanent neonatal diabetes (PND) who had inherited the I1425V mutation in a dominant fashion. The same dominant mutation has also been observed in another patient also with PND (29). Functional analysis of I1425V showed that it over-activated the K\textsubscript{ATP} channel complex by increasing the Mg\textsuperscript{2+} nucleotide-dependent stimulatory action of SUR1 on the pore, which is consistent with activating mutations in ABCC8 causing neonatal diabetes. Nevertheless, it is remarkable that substituting the same isoleucine residue to leucine (as we have done in this study) is shown to cause CHI clinically and not PND. This phenomenon has been seen with regards to another mutation (30).

Membrane expression WT SUR1 and K\textsubscript{ATP}6.2-GFP

The trafficking analysis of all the SUR1 mutants once again highlighted the difference between NBD2 and TMD mutants. The co-expression of K\textsubscript{ATP}6.2-GFP with WT SUR1 shows clear membrane expression of K\textsubscript{ATP}6.2 in contrast to a predominantly ER localization when expressed alone. This is consistent with the proposed role of SUR1 shielding the ER-retention signals on K\textsubscript{ATP}6.2 (31).

The NDB2 mutants (D1506E, M1514K, G1485E and I1425L) resulted in clear translocation of K\textsubscript{ATP}6.2-GFP from the ER to the plasma membrane suggesting a functional deficit. In fact, it is possible that the mutant channels may be expressed better at the membrane than WT K\textsubscript{ATP} channels. This observation has been previously reported by the others (15), where a total of 13 missense mutations causing DZX unresponsive disease were studied, and this included mutations in TMD1, NBD1 and NBD2. They found that all the mutants were expressed at the membrane.

However, here we find that the TMD mutants in general, show a greater retention in the ER. This has not been reported previously for dominant mutations (14). Trafficking defects have been observed only in homozygous recessive mutations seen in the ABCC8 and KCNJ11 genes, which cause a severe form of CHI. The dominant DZX unresponsive mutations studied here also cause severe CHI. The G92D and A113V mutants are located in the TMD0 of the SUR1 protein, and this region has been previously implicated in physically associating with the Kir6.2 subunit. This coupling aids the trafficking of the channel to the membrane, hence mutations in this region may affect channel complex formation.

Paternal mutation carriers

The paternal mutation carriers reported in this study are asymptomatic with no hypoglycaemia. This lack of disease phenotype has been observed in previous reports on dominant CHI mutation carriers (13). One possible explanation is reduced penetrance, which is a feature observed in autosomal dominant disease conditions, such as familial cancer syndromes (for a review see (32)). The causes of reduced penetrance may be due to a combination of unknown genetic, environmental and lifestyle factors. In the case of CHI we are not certain of the mechanism. For example, variable expression of the mutant allele in different family members could lead to changes in the strength of the dominant-negative effect.

It is likely that paternal heterozygous mutations causing diffuse disease CHI are acting through a different mechanism. The possible existence of a second mutation in the maternal allele cannot be disregarded, as it may have gone undetected by the initial genetic analysis; alternatively there could be a mutation in a non-ABCC8 non-KCNJ11 gene, which cannot be ruled out as the genetic make-up of ~50% CHI patients is still unknown (22).

Conclusions

A question raised by this study is; why do these dominant mutations behave so differently from other known dominant mutations which are responsive to medical therapy? It may be due to reduced penetrance, which can influence the effect of a genetic mutation. This study also suggests that the location of the mutation is significant when it comes to the mechanism of disease pathogenesis. Our data clearly suggest that mutations in the NBD completely abolish the channels response to conventional drugs such as DZX without affecting the surface expression of the channel. The mutations within the SUR1 TMD, however, show increased channel retention in the ER. The functional work suggests that the channels are responsive to some degree to K\textsubscript{ATP} channel activators, although the response may be inadequate to control the hypoglycaemia observed in the patients.

A detailed understanding of the disease mechanisms will potentially allow one to tailor patient therapy based on the individual mutation. For example, cystic fibrosis is a life-threatening hereditary disease caused by mutations in the Cystic fibrosis transmembrane regulator (CFTR) gene. This condition is characterized by thick mucus secretions in the lungs and intestines which impairs the function of the organ, and ultimately leads to death in most affected individuals by the age of 30 (23). A common mutation in CFTR (AF508) prevents the protein from exiting the ER (33), hence this protein has been extensively studied to develop clinically useful therapies. This research has identified small molecules which can initiate cellular responses, such as improving the protein folding capacity of the cell or acting as a highly specific pharmacological chaperone for misfolded proteins (27).

Ivacaftor is a mutation-specific drug which is currently in use to treat cystic fibrosis (34). The drug has already had a great medical impact in improving patient lives (35). It acts as a potentiator to improve channel gating and has recently received approval from the US Food and Drug Administration (FDA) for use in treating patients with the G551D-CFTR mutation as well as eight others (35). Carbamazepine, an anti-epileptic drug has also been identified as a novel, clinically useful corrector of the CFTR protein. This drug has also been shown to improve the surface expression of K\textsubscript{ATP} channels, although it is only effective in ABCC8 mutations in the TMD0 region (27). A better understanding of the mechanisms underlying disease in CHI patients opens up the possibility of ‘correcting’ TMD0 mutants in CHI patients and this could lead to a future where these patients no longer require a pancreatectomy.

Materials and Methods

Histology

Haematoxylin and eosin (H & E) staining was used on paraffin sections (3 µm thick) which were prepared from blocks of resected pancreatic tissue from the probands that had a
pancreatectomy. Microsatellite analysis of chromosome 11p15 was also carried out on the available pancreatic tissue.

**Genetic diagnosis of patients**

Genomic DNA was extracted from peripheral leukocyte cells and also from pancreatic tissue, using standard procedures. The KCNJ11 gene was initially sequenced in the patients, and in all cases there was no mutation found. This lead to the sequence analysis of the much larger ABCC8 gene, which was compared with the published sequence, NM_000352.2. This sequence incorporates the alternatively spliced residue in exon 17. If an ABCC8 mutation had been identified in the proband then the parents were also subject to mutation testing. Upon finding a de novo mutation, microsatellite analysis was used to establish family relationships as previously shown (16). Novel mutations were established by sequencing 500 control chromosomes which did not show the mutations. In addition to this, dosage analysis was carried out in all probands (MRC Holland, Amsterdam, the Netherlands, MLPA, kit P117), which failed to show a second

**Molecular biology**

The QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used to create single point mutations into the hamster SUR1 clone pcDNA3 (accession number L40623) or mouse Kir6.2 clone pcDNA3.1/Zeo (accession number D50581). The presence of mutations was confirmed by Sanger sequencing (16).

**Cell culture and transfections**

Human Embryonic Kidney 293 (HEK293) cells were grown in Minimum Essential Medium (MEM) with Earle’s salt and glutamine (PAA), which was supplemented with (1%) penicillin-streptomycin (Life technologies) and (10%) Fetal Bovine Serum (Life technologies). HEK293 cells were sub cultured when they were ~90% confluent for all the biochemical and functional work. For homologous expression studies a total of 2 μg of WT/mutant SUR1 was transfected in HEK293 cells with 500 ng Kir6.2, in addition to 200 ng pDsRed2-ER transfected in HEK293 cells. Quantitative co-localization analysis was performed on the images acquired. The fraction of green pixels (Kir6.2-GFP) which also contained red pixels (pDsRed2-ER) was measured and calculated as a percentage. ImageJ and the plugin JACoP was used to analyse the confocal images. The co-localization co-efficient M1 and M2 provide a representation regarding the amount of co-localizing objects in the components of an image (36). M1 is for channel 1, and this is sum of all the green pixels which are red, the values of which have been presented in this paper. For display, the images were filtered at a median range, with a radius of 2.0 pixels and converted to TIFFs.

**Electrophysiology**

HEK293 cells were seeded into six wells plates, and transiently transfected at ~90% confluence. All experiments were performed in triplicate. Upon successful transfection (>50%) the cells were loaded with 0.037 MBq/ml of 86Rb+, and incubated for 24 h in optimum conditions (5% CO2, 37°C). Subsequently, the cells were washed three times with 2 ml of hepes buffered saline (HBS) assay medium which consisted of (in mm) 10 HEPES, 10 anhydrous glucose, 130 NaCl, 7 KCl, 2 CaCl2 and 1 MgCl2. Cells were incubated for 5 min with 2 ml of HBS medium with or without channel stimulants/inhibitors. The 5 min incubation was used on the following four drug conditions: DMSO, 100 μM DZX, 100 μM diazoxide plus 10 μM glibenclamide, and 2.5 mM NaN and 20 mM 2-deoxy-D-glucose and 10 μM glibenclamide. Cells were incubated for 15 min under MI which was achieved using 2.5 mM NaCN and 20 mM 2-deoxy-D-glucose. After incubation, the supernatant was aspirated into 6 ml polyethylene scintillation vials for counting. Cells were then lysed with 2% Triton/ HBS solution and the cell lysates were measured in liquid scintillation vials. All vials were assayed for the 86Rb+ content by measuring the Cherenkov radiation in a liquid scintillation counter (Tricarb, Packard 2000CA). All data generated have been analysed using one-way ANOVA, followed by the Bonferroni multiple comparison test to determine statistical significance.

**86Rb+ efflux assay**

HEK293 cells were seeded on to 10 mm coverslips in 6-well plates, and used for patch-clamp experiments 48–72 h post-transfection. For whole-cell patch-clamp, the ICS had the following composition (mm): 107 KCl, 1.2 MgCl2, 1 CaCl2, 10 EGTA and 5 HEPES with 0.1 MgATP and 1 NaADP, pH 7.2 using KOH. The extracellular solution (ECS) for whole-cell recordings contained (mm): 140 NaCl, 5 KCl, 1.2 MgCl2, 2.6 CaCl2, 10 HEPES, at pH 7.4 using NaOH. The composition of the 140K+ bath and pipette solution for inside-out patch-clamp recordings was; (mm) 140 KCl, 10 HEPES, 1 EGTA, 1 MgCl2, pH 7.3 with KOH. The bath solution and the drugs were perfused onto cells using a gravity driven perfusion system. Patch pipettes were pulled from borosilicate glass with a resistance of ~2–4 MΩ for whole-cell recordings and 5–8 MΩ for single channel recordings. All the electrophysiological recordings were made at room temperature. Capacitance transients and series resistance in whole-cell recordings was compensated electronically by using amplifier circuitry (Multi-camp 700B). Whole-cell currents were recorded using a ramp protocol (−150 to +50 mV for 1 s). The data were sampled at 5 kHz and filtered at 1 kHz with the filter provided in the Multi-camp 700B (4 Pole Bessel). Signals were converted from analogue to digital signals by Digidata 1440A (Molecular Devices). Recordings were acquired and analysed using pClamp 10 and Clampfit 10 (Axon Instruments), respectively.
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References


