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Functional characterization of activating mutations in the sulfonylurea receptor 1 (*ABCC8*) causing neonatal diabetes mellitus in Asian Indian children

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Abstract

Background: Gain-of-function of ATP-sensitive K^+ (K_{ATP}) channels because of mutations in the genes encoding SUR1 (*ABCC8*) or Kir6.2 (*KCNJ11*) is a major cause of neonatal diabetes mellitus (NDM). Our aim is to determine molecular defects in K_{ATP} channels caused by *ABCC8* mutations in Asian Indian children with NDM by in vitro functional studies.

Methods: Wild-type (WT; NM_000352.4) or mutant sulfonylurea receptor 1 (SUR1) and Kir6.2 were co-expressed in COSm6 cells. Biogenesis efficiency and surface expression of mutant channels were assessed by immunoblotting and immunostaining. The response of mutant channels to cytoplasmic ATP and ADP was assessed by inside-out patch-clamp recordings. The response of mutant channels to known K_{ATP} inhibitors in intact cells were determined by ^{86}Rb efflux assays.

Results: Five SUR1 missense mutations, D212Y, P254S, R653Q, R992C, and Q1224H, were studied and showed increased activity in MgATP/MgADP. Two of the mutants, D212Y and P254S, also showed reduced response to ATP^{4-} inhibition, as well as markedly reduced surface expression. Moreover, all five mutants were inhibited by the K_{ATP} channel inhibitors glibenclamide and carbamazepine.

Conclusions: The study shows the mechanisms by which five SUR1 mutations identified in Asian Indian NDM patients affect K_{ATP} channel function to cause the disease. The reduced ATP^{4-} sensitivity caused by the D212Y and P254S mutations in the L0 of SUR1 provides novel insight into the role of L0 in channel inhibition by ATP. The results also explain why sulfonylurea therapy is effective in two patients and inform how it should be effective for the other three patients.

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Keywords

activating mutation; *ABCC8*; *KCNJ11*; sulfonylurea; neonatal diabetes mellitus

1 | INTRODUCTION

ATP-sensitive K^+ (K_{ATP}) channels couple cell metabolism to membrane excitability in many tissues including the heart, brain, skeletal muscle, and endocrine cells.[1–3] In glucose-sensing cells, such as pancreatic islet β-cells, they couple changes in blood glucose to cell activation. The pancreatic K_{ATP} channel is an octameric complex of four pore-forming inwardly rectifying potassium channel Kir6.2 subunits and four regulatory sulfonylurea receptor 1 (SUR1) subunits.[1,3] K_{ATP} channels are regulated by the relative concentrations of intracellular ATP and ADP, which enables them to regulate membrane potential according to the energetic state of the cell, and thereby mediating glucose-stimulated insulin secretion.[1] Activating (gain-of-function) mutations in the genes encoding Kir6.2 (*KCNJ11*) or SUR1 (*ABCC8*) uncouple plasma glucose from insulin secretion and cause neonatal diabetes mellitus (NDM).[4,5] Many of these mutations result in NDM alone, but some can cause a more severe phenotype in which developmental delay, muscle weakness and epilepsy accompany neonatal diabetes, a condition known as DEND syndrome.[4]

NDM is a rare (1 in 100 000-200 000), usually monogenetic, non-autoimmune form of diabetes that occurs typically in the first 6 months of life with reduced insulin secretion resulting in hyperglycemia and low birth weight.[5,6] NDM may be either permanent (PNDM), requiring life-long treatment, or transient (TNDM), in which the diabetes may spontaneously remit within 1 to 18 months, but often relapse to diabetes during adolescence or early adulthood.[7–9] Genetic defects in at least 20 different genes have been reported to cause neonatal diabetes.[6] Activating mutations in the K_{ATP} channel encoding genes (*ABCC8* and *KCNJ11*) are the most common genetic causes of NDM in various populations, accounting for ~50% of all cases.[4,6] To date, more than 200 different point mutations in *ABCC8* and *KCNJ11* genes have been reported in the human gene mutation database (HGMD) to cause various forms of NDM and DEND syndrome. In some patients, K_{ATP} channel inhibitors such as sulfonylureas have been used successfully to suppress mutant channel activity and restore glycemic control.[4,6,10]

Here, we describe the structural and functional cause of heterozygous SUR1 (*ABCC8*, NM_000352.4) mutations in five different genetically unrelated Asian Indian children associated with NDM or with some neurological features. Of the five mutations characterized in this study, two (D212Y and R653Q) have been previously reported by our group.[11] The P254S mutation has been reported in an Indian patient by Flanagan et al.[12] The R992C and Q1224H mutations have not been reported in Asian Indian children so far. Functional characterization has not yet been described for any of these mutations.

2 | MATERIALS AND METHODS

2.1 | Clinical subjects

The study group is comprised of children with neonatal diabetes and syndromic forms of diabetes referred to Dr. Mohan's Diabetes Specialties Centre, a large diabetes hospital in south India. All probands included in the study were negative for pancreatic auto-immune antibodies [glutamic acid decarboxylase auto antibodies (GAD) and islet antigen 2 auto antibodies (IA2)]. Diabetes is defined by random plasma glucose greater than 200 mg/dL on more than one occasion for these patients and the onset of diabetes was on or before 6 months of age.

NDM children carrying the D212Y or the R653Q mutation in SUR1 encoding gene *ABCC8* have been described previously.[11] The remaining three mutations, P254S, R992C, and Q1224H in SUR1 were recently identified. Clinical and genetic information of Asian Indian NDM children harboring the *ABCC8* (NM_000352.4) gene mutations are listed in Table 1. The study was approved by the local institutional ethical committee of the Madras Diabetes Research Foundation (MDRF), India. Written informed consent was obtained from all the parents or guardians of the children before the study. The reported investigations have been carried out in accordance with the Declaration of Helsinki principles as revised in 2000.

2.2 | In silico and structural analysis of NDM mutations

Prior to the availability of high resolution K_{ATP} channel structures, online computational algorithms such as SIFT (Sorting Intolerant from Tolerant), Polyphene-2 (Polymorphism Phenotype), and Mutation taster were used to predict the possible effects of the five amino acid substitutions (D212Y, P254S, R653Q, R992C, and Q1224H) on SUR1 protein function. Following our recent publication of the K_{ATP} channel structure obtained by single-particles cryo-electron microscopy (EM),[13,14] we were also able to map three of the five mutated residues which are resolved in our structure including D212, P254, and Q1224 (see Section 3).

2.3 | Plasmids and transfection

Human *ABCC8* cDNA (NM_000352.4) tagged with a FLAG epitope (DYKDDDDK) at the N-terminus was cloned into pCMV6b and human *KCNJ11* cDNA (NM_000525.3) was cloned into pCDNA3 for this study. Point mutations were introduced in desired positions using site-directed mutagenesis PCR according to the manufacturer's instructions (Agilent Technologies). SUR1 cDNA and Kir6.2 cDNA were co-transfected into COSm6 (kidney cell line derived from African green monkey) at 70% to 80% confluency using FuGENE 6 transfection reagent (Promega).

2.4 | Western blotting

Thirty-six to 42 hours post-transfection, cells were lysed and total proteins were prepared with a buffer containing 20 mM HEPES, pH 7.0, 5 mM ethylenediaminetetraacetic acid (EDTA), 150 mM NaCl, 1% Nonidet P-40) containing complete protease inhibitors (Roche). 25 to 30 μg of protein lysates were loaded and run on 7.5% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred onto nitrocellulose membrane. The

membrane was blocked with 5% non-fat dry milk solution for 2 hours at room temperature. WT and mutant SUR1 proteins were detected with rabbit anti-SUR1 (1:400) primary antibody (raised against the C-terminus of SUR1, KDSVFASFVRADK)[15] and Horse-radish peroxidase (HRP) conjugated donkey anti-rabbit IgG (1:10000) secondary antibodies. Finally, membranes were developed with the super signal femto ECL kit (Thermofisher, USA), images were captured and analyzed using the FluorChemE system (ProteinSimple, USA).

2.5 | Immunofluorescence staining

COSm6 cells were transfected as described above and 24 hours later plated on glass coverslips for staining. For detecting surface channels, cells were washed twice with ice cold phosphate buffered saline (PBS) and incubated with 10 μg/mL M2 anti-FLAG antibody (Sigma, USA) in Opti-MEM plus 0.1% BSA at 4°C for 1 hour. Cells were then washed twice in ice cold PBS + 0.1% BSA and fixed with −20°C methanol for 10 minutes on ice. Fixed cells were incubated with Alexa 488 conjugated goat anti-mouse secondary antibody (1:1000) for 1 hour at room temperature, followed by 3 × 10 min washes with PBS + 0.1% BSA and 1 × 10 min wash in PBS. Coverslips were mounted in mounting medium containing DAPI to stain the nuclei. To stain for total cellular SUR1, cells were fixed in methanol first before incubation with primary and secondary antibodies as above. Fluorescence images were acquired using a Zeiss LSM780 confocal microscope.

2.6 | Electrophysiology

The activity of WT and mutant channels in solutions containing defined ATP or ADP concentrations with or without Mg^{2+} were assessed using inside-out patch-clamp method described previously.[16,17] COSm6 cells were transfected with cDNAs for WT or mutant SUR1, along with Kir6.2 and enhanced green fluorescent protein (eGFP), which facilitates identification of transfected cells. Twenty-four hours after transfection cells were plated onto cover-slips, and patch-clamp recordings were performed in the following 2 days. Micropipettes were pulled from non-heparinized Kimble glass (Fisher Scientific) with resistance typically ~2 to 5 MΩ when filled with K-INT (see below). Inside-out patches were voltage clamped with an Axopatch 1D amplifier (Axon Inc.). The bath (intracellular) and pipette (extracellular) solution (K-INT) contained 140 mM KCl, 10 mM HEPES, and 1 mM EGTA (pH 7.2). For measuring the ATP^{4-} sensitivity of WT, D212Y, and P254S channels, 1 mM EDTA was added to the K-INT solution to chelate any residual contaminating Mg^{2+}. All currents were measured at a membrane potential of −50 mV. Data were analyzed using the pCLAMP 8.0 software (Axon Instrument).

2.7 | $^{86}Rb^{+}$ efflux assay

$^{86}Rb^{+}$ efflux assays were used to test K_{ATP} channel response to glibenclamide and carbamazepine in intact cells as described previously.[16] COSm6 cells were transfected with Kir6.2 along with WT or mutant SUR1 for ~24 hours and then incubated with $^{86}RbCl$ (1 μCi/mL) overnight. The next day, cells were washed once in Krebs Ringer's solution and then incubated for 30 minutes in Ringer's solution containing $^{86}RbCl$ (1 μCi/mL) and metabolic inhibitors (1 mM 2-deoxy-D-glucose and 2.5 μg/mL oligomycin) to activate the channels before efflux measurements in the presence of 1 μM glibenclamide, 50 μM

carbamazepine, or 0.1% dimethyl sulfoxide (DMSO) vehicle control in the presence of metabolic inhibitors in Ringer's solution. To measure efflux, the solution was collected and replaced with fresh solution at selected time points (2.5, 5, 7.5,15, 25, and 40 minutes). At the end of a 40-min period, cells were lysed in Ringer's solution containing 1% SDS. $^{86}Rb^{+}$ in the solutions collected at each time point and the cell lysate was counted in a scintillation counter. The percentage of efflux at each time point was calculated as the cumulative counts in the solution for a given time point divided by the total counts from all time points and the cell lysate.

2.8 | Statistical analysis

Results were expressed as means ± SEM. Differences between WT and mutants were tested using two-tailed unpaired student's *t* test; '*P*' values of <0.05 were considered statistically significant.

3 | RESULTS

3.1 | Mutations and clinical data

Five different *ABCC8* missense mutations were identified in five genetically unrelated Asian Indian children diagnosed with NDM, and in one child some neurological symptoms were also noted. Detailed clinical and genetic information on patients carrying NDM mutations is listed in Table 1. Four of these mutations, including D212Y, R653Q, R992C, and Q1224H are heterozygous in the patients and de novo in nature, while the P254S mutation is homozygous in the patient with a mutant allele inherited from each of the parents. Of the five mutations included in this study, two (D212Y and R653Q) have been previously reported by our group,[11] P254S has been reported by another group from a different patient,[12] and two (R992C and Q1224H) are novel. No functional characterization has been carried out for any of the mutations.

The mutation D212Y was identified in a child with 2.5 kg birth weight and diabetes onset at 60 days of age.[11] The child presented with elevated blood glucose of 235 mg/dL and Hemoglobin A1c (HbA1c) of 53 mmol/mol (7%). Developmental delay was not noted but he had neonatal hepatitis and sepsis. The mutation P254S was detected in a child with PNDM who had birth weight of 2.8 kg with diabetes diagnosed at 90 days of age; he presented with high blood glucose of 570 mg/dL and HbA1c of 113 mmol/mol (12.5%). Interestingly, the child is homozygous for this mutation which is present as a heterozygous mutation in both parents. Although neither parent had been diagnosed with NDM, both parents exhibited impaired glucose tolerance (IGT), with the father's fasting blood sugar at 118 mg/dL and the mother's fasting blood sugar at 108 mg/dL on a recent clinical visit. The child with the R653Q mutation was diagnosed with TNDM at 96 days of age. He had normal birth weight of 3.2 kg, and presented with high blood glucose of 774 mg/dL and a HbA1c level of 55 mmol/mol (7.2%). In addition, developmental delay was noted in this child.[11] The R992C mutation was identified in a child with birth weight of 2.5 kg and onset of diabetes at 90 days of age. This child presented with high blood glucose of 527 mg/dL with a corresponding HbA1c level of 61 mmol/mol (7.7%). The child carrying the Q1224H mutation exhibited hyperglycemia with the onset of diabetes at 90 days of age. He had birth

weight of 2.5 kg with high blood glucose of 450 mg/dL and a corresponding HbA1c level of 63 mmo/mol (7.9%). Of note, this child harbored a compound heterozygous mutation in the *INS* (NM_0.000207.2) gene promoter (c.−176C > T), in addition to the *ABCC8* mutation.

All five children were on insulin treatment to maintain their glycemic levels. The patient with the D212Y mutation was switched from subcutaneous insulin injection to oral sulfonylurea therapy at the age of 15 months. His blood glucose level was maintained at a normal level, lowering from 235 to 100 mg/dL after sulfonylurea transfer.[11] The patient with the R992C mutation was also transferred to sulfonylurea therapy under his clinicians' discretion and has responded well with good glycemic control.

3.2 | In silico and structural analysis of *ABCC8* gene mutations

SUR1 belongs to the ABC transporter protein superfamily.[1] It has an ABC core structure consisting of two transmembrane domains TMD1 and TMD2 each followed by a nucleotide binding domain, NBD1 and NBD2; in addition, it has an N-terminal transmembrane domain called TMD0 that is linked to the ABC core by a cytoplasmic linker referred to as L0 (Figure 1B). We initially assessed the possible functional impact of these missense mutations or variants using three commonly used online programs: Polyphene2, SIFT, and Mutation taster. All five mutations were predicted to affect protein function or to be disease-causing by the three programs. Furthermore, multiple sequence alignment (MSA) and evolutionary conservation analysis showed close conservation of the affected amino acids between related species such as human, rat, mouse and hamster, indicating that these residues are important for normal protein function (Figure 1A).

Following our recent publication of the K_{ATP} channel structure,[13,14] we were able to map some of the mutated SUR1 residues in the 3D structure (PDB: 6BAA). Mutations D212Y and P254S are both located in the cytoplasmic linker "L0", which connects the N-terminal transmembrane domain TMD0 to the ABC core structure of SUR1 (Figure 1C). The other three mutations, R653Q, R992C, and Q1224H are located in the ABC core of SUR1. Q1224H is situated between the fifteenth and sixteenth transmembrane helices in TMD2 and is modeled in the structure (Figure 1C); however, R653 and R992 are each in flexible linkers that precede and follow the first nucleotide binding domain (NBD1), respectively, and which are not clearly visible in the cryoEM density map and therefore not modeled.[13,14]

3.3 | Functional analysis of *ABCC8* (SUR1) mutations

To assess the molecular effects of these mutations on the channel, we first determined the processing efficiency of mutant SUR1 by Western blotting. SUR1 harbors two N-linked glycosylation sites that undergo complex glycosylation in medial Golgi to yield mature SUR1 that migrates slower as an upper band, in contrast to the immature core-glycosylated SUR1 in the ER/early Golgi that migrates faster as a lower band on Western blots.[18] Because only fully assembled SUR1/Kir6.2 complex can exit the ER and reach the medial Golgi, the presence and abundance of the complex glycosylated SUR1 band also reflects channel biogenesis efficiency.[15,18] Lysates from cells expressing WT SUR1 and Kir6.2 or mutant SUR1 and Kir6.2 were analyzed using an anti-SUR1 antibody.[15,19] As shown in Figure 2A, the R653Q, R922C, and Q1224H SUR1 mutants all had clear lower and

upper bands as was the case for WT SUR1; by contrast, the D212Y and P254S SUR1 mutants were seen mostly as the lower immature form with barely detectable upper band that corresponds to the mature protein. To better quantify the extent to which each mutation impacts SUR1 protein level and maturation, densitometry of the bands was performed using Fiji ImageJ.[20] Results showed that the total SUR1 protein intensity was not significantly altered by any of the mutations (Figure 2B). However, the mature upper SUR1 band intensity relative to the total SUR1 protein intensity was significantly reduced in D212Y, P254S, and R922C, although the extent of reduction in R992C was much less compared to D212Y and P254S (Figure 2C). These observations indicate that D212Y and P254S severely impair SUR1 maturation and likely expression at the plasma membrane. Immunofluorescence staining of surface K_{ATP} channels confirmed that while R653Q, R992C and Q1224H mutants were clearly present like WT channels, expression of the D212Y and P254S mutants was markedly reduced (Figure 3). Staining of permeabilized cells for total SUR1, however, showed abundant protein levels for all five mutants similar to WT, indicating that the majority of the D212Y and P254S mutant SUR1 proteins were trapped inside the cell (Figure 3).

Intracellular ATP and ADP are two key physiological ligands that determine the net activity of K_{ATP} channels. ATP and, to a much lesser extent, ADP can inhibit channel activity via Kir6.2 and this inhibition does not require Mg^{2+}. On the other hand, ATP and ADP can stimulate channel activity, in a Mg^{2+}-dependent way, by interacting with and causing dimerization of the nucleotide binding domains of SUR1.[3] In physiological conditions, the relative change in ATP and ADP concentrations as blood glucose levels fluctuate will tip the balance towards ATP inhibition or MgATP/MgADP stimulation to dictate channel activity. Reduced sensitivity to ATP inhibition or enhanced sensitivity to MgATP/MgADP stimulation have been reported to be the common mechanisms underlying NDM caused by mutations in *KCNJ11* or *ABCC8*, respectively.[4,21,22] We therefore determined whether the five SUR1 mutations in our study alter the MgATP/MgADP sensitivity of the channel by comparing channel activity in 0.1 mM MgATP alone vs 0.1 mM MgATP plus 0.5 mM MgADP using inside-out patch-clamp recordings (see Section 2), a scheme that is highly sensitive in detecting altered MgATP/MgADP response.[17,23] We found that mutant channels formed by Kir6.2 and SUR1 with the D212Y mutation or the P254S mutation had markedly increased activity in both 0.1 mM MgATP and 0.1 mM MgATP plus 0.5 mM MgADP compared to WT channels, with the D212Y mutant being the most active (Figure 4A). In comparison, the changes associated with the R653Q, R992C and Q1224H SUR1 mutations were less pronounced. No significant differences were observed in currents detected in 0.1 mM MgATP for all three mutations. However, currents in 0.1 mM MgATP plus 0.5 mM MgADP were significantly higher for all three compared to WT (Figure 4B). These results indicate that all five mutations increase channel activity, by augmenting Mg-nucleotide stimulation.

The majority of the NDM-causing *ABCC8* mutations reported to date enhance channel function by increasing channel response to MgATP/MgADP stimulation without affecting ATP inhibition.[4,17,24] However, because the activity of the D212Y and the P254S mutants was much greater than WT in 0.1 mM MgATP, we wondered whether the two mutations may also render the channel less sensitive to ATP inhibition, which is mediated primarily

through the Kir6.2 subunit. To test this, we performed additional patch-clamp recording experiments to determine the sensitivity of these mutants to ATP inhibition (Figure 5A). Because MgATP can both inhibit and stimulate channel activity by interacting with Kir6.2 and SUR1 respectively,[1,3,25] in order to eliminate the MgATP stimulation effect, 1 mM EDTA was added to K-INT solutions containing various concentrations of ATP to chelate any residual Mg^{2+}[26] (referred to as ATP^{4-}). We found that the activity of both the D212Y and the P254S mutants were significantly higher than WT channels at all three ATP^{4-} concentrations tested (Figure 5B). Worth noting is that the difference between the two mutants and the WT at 0.1 mM MgATP (Figure 4B) was greater compared to the difference observed in 0.1 mM ATP^{4-} (Figure 5B), which reveals that in addition to reducing ATP^{4-} sensitivity both D212Y and P254S also increase channel response to MgATP/MgADP stimulation (see Section 4).

NDM-causing mutations in *KCNJ11* in most cases manifest as dominant heterozygous mutations, but in *ABCC8* both dominant and recessive mutations have been reported.[4] Interestingly, the P254S proband was homozygous for the mutation, with both parents being heterozygous for the mutation. To test whether the mutant can impact overall channel function when expressed in a simulated heterozygous condition, we compared the response of channels from cells co-expressing WT and the P254S mutant SUR1 at a 1:1 ratio together with Kir6.2 (WT:P254S) to those from cells expressing pure WT or P254S channels. We found that the WT:P254S channels had a significantly higher activity in 0.1 mM MgATP plus 0.5 mM MgADP than WT channels (Figure 4B), although the increase was much smaller ($P = 0.03$ compared to WT) than that observed for pure P254S channels ($P < 0.00001$ compared to WT). This subtle increase is in line with the clinical observation of the heterozygous parents who appeared to be glucose intolerant with fasting blood glucose higher than normal.

3.4 | Response of mutant channels to K_{ATP} channel inhibitors

NMD patients carrying activating K_{ATP} mutations can in many cases be successfully treated with sulfonylureas which are oral hypoglycemic drugs that inhibit K_{ATP} channel activity, resulting in good glycemic control without the need for insulin injection.[4,6,10,27–29] We therefore tested whether the mutant K_{ATP} channels are responsive to sulfonylurea inhibition. To maximize detection of inhibition, we performed $^{86}Rb^{+}$ efflux experiments with cells that were pretreated with metabolic inhibitors (see Section 2), a condition that lowers intracellular ATP/ADP ratios to activate K_{ATP} channels. As shown in Figure 6, all mutant channels were significantly inhibited by 1 μM glibenclamide (GBC) as seen for WT channels, indicating their overactivity in vivo would be suppressed and insulin secretion restored by sulfonylurea administration in the patients. In addition to sulfonylureas, carbamazepine (CBZ), a commonly prescribed anticonvulsant was recently found to be a potent K_{ATP} channel inhibitor.[30–32] Because CBZ is already being used to treat epilepsy, it may be useful for treating NDM patients with neurological symptoms (DEND patients). Therefore, mutant channels were also tested for their response to CBZ. Similar to GBC, CBZ (at 50 μM) likewise inhibited all five mutant channels as well as WT channels, suggesting this drug may also be effective in treating pathology caused by the activating mutations in SUR1 described here.

4 | DISCUSSION

In this study, we characterized the impact of five SUR1 mutations which were identified from Asian Indian children diagnosed with NDM on K_{ATP} channel function. By analyzing the processing efficiency and surface expression of the mutant channels and their response to intracellular ATP and ADP, we conclude that all five mutations altered channel response to intracellular nucleotides to enhance channel activity, and that two mutations, D212Y and P254S, also severely impaired channel biogenesis and surface expression. Below we discuss the implications of our findings for disease mechanisms, channel structure-function relationships, and therapeutic strategies.

Although all five mutations resulted in increased channel activity in MgATP/MgADP, the D212Y and P254S mutations had by far the largest effects. Paradoxically, D212Y and P254S also drastically reduced channel surface expression, which is expected to reduce channel function. These observations suggest that the overactive defects of the D212Y and P254S mutants because of altered nucleotide sensitivities outweigh their biogenesis and surface expression defects to result in an overall gain-of-channel-function effect and the NDM disease phenotype. Many *ABCC8* and *KCNJ11* mutations have been shown to affect both channel gating and expression, such as E208K and V324 M in SUR1[17] as well as Q52R, V59G, R201C, R201H, and I296L in Kir6.2[33] identified in NDM patients, and R74W and E128K in SUR1 identified in patients with congenital hyperinsulinism. For these mutations, the disease phenotype is the outcome of the combined effects of a mutation on K_{ATP} channel surface expression and the open probability of the channel in the plasma membrane. Such complex mechanisms can only be gleaned from a detailed functional characterization as performed in this study. Of the five mutations, P254S stands out as the only mutation homozygous in the patient, resulting from consanguineous marriage of parents each heterozygous for the mutation. The same mutation has been reported recently in a different consanguineous Indian family, although clinical information on the heterozygous parents was not reported.[12] Previous genetic studies of PNDM mutations in *KCNJ11* and *ABCC8* have shown that *KCNJ11* mutations are in most cases dominant heterozygous mutations in patients, while *ABCC8* mutations can be dominant or recessive.[4] In our study, neither parents of the P254S proband had records of NDM, but recent clinical visits showed that both had impaired glucose tolerance with fasting blood glucose above 100 mg/dL (see Section 3). This is consistent with our functional data showing higher activity of channels formed by co-expression of the P254S mutant and the WT in MgATP/MgADP compared to WT (Figure 4B). It is possible that mild diabetes disease in the parents was missed when they were infants. Alternatively, heterogeneous diabetes clinical phenotype in family members with the same gain-of-function *ABCC8* mutation has been reported recently.[34] Finally, with regard to functional data-disease phenotype correlation, it should be noted that the patient carrying the Q1224H mutation is compound heterozygous of another mutation in the *INS-1* promoter, which too may contribute to the disease.

The residues mutated in this study are all highly conserved (Figure 1A). The two mutations D212Y and P254S that cause the most severe defects in channel sensitivity to ATP and ADP are both located in the “L0” linker connecting TMD0 with the ABC core of SUR1 (Figure 1). This linker is critical for the interaction between SUR1 and Kir6.2,[35,36] and

is likely involved in transducing the conformational change in the ABC core induced by Mg-nucleotides to the Kir6.2 pore to stimulate channel activity.[13,14] A number of mutations in this linker have already been reported in patients with NDM, including E208K[17] and L225P.[24] Somewhat unexpectedly, in addition to enhancing MgATP/MgADP response, both D212Y and P254S mutant channels also exhibited substantially increased activity in ATP^{4-} in comparison to the WT channel. This is in contrast to other L0 mutations such as the aforementioned E208K and L225P which were not reported to have an effect on channel sensitivity to ATP inhibition.[17,24] Recent cryo-EM structures of the K_{ATP} channel bound to ATP in Kir6.2 show that while the ATP molecule is primarily coordinated by residues in the N- and C-terminus of adjacent Kir6.2 subunits, L0 of SUR1 is in close physical proximity to the ATP binding pocket in Kir6.2[13] (Figure 1D). Mutagenesis and functional studies also support a role for L0 in regulating channel sensitivity to ATP inhibition.[37] The profound effects of D212Y and P254S on ATP inhibition suggest these residues are likely involved in facilitating ATP binding or subsequent conformational change that leads to channel closure. Precisely how these two mutations reduce ATP inhibition while others in L0 do not remain interesting future questions. Collectively, our findings here further underscore the importance of L0 in channel regulation by intracellular nucleotides as well as channel biogenesis. The other three mutations R653Q, R992C, and Q1224H are located in the ABC core of SUR1, but only Q1224 has sufficient signal in the electron microscopy density map to be modeled in the recently published high resolution K_{ATP} channel structure.[13] Q1224 is situated between the fifteenth and sixteenth transmembrane helices in TMD2 and may be involved in linking conformational changes at the nucleotide binding folds to the transmembrane helices.

An important goal of functional characterization of NDM-associated K_{ATP} mutations is to assess whether channel overactivity caused by the mutations can be suppressed by sulfonylureas such that patients may be transferred from insulin injection to oral sulfonylurea therapy. Our data show that all mutations are effectively inhibited by glibenclamide (or glyburide). Consistent with this finding, the patient with the D212Y mutation has shown good response to oral sulfonylurea therapy after switching from insulin injection at the age of 15 months. Another mutation, R992C associated with a PNDM patient also showed good response to sulfonylurea therapy. Our results serve as the basis for transferring other patients to sulfonylurea therapy. Following our functional studies, the other three probands have also been advised to consider sulfonylurea therapy. The proband with the P254S mutation is scheduled to start the trial on his next clinical visit. The patient with the R653Q mutation was diagnosed at the age of 7 and is now 20 years old doing well on insulin therapy. The clinician and patient will discuss the possibility of switching to sulfonylurea therapy in the near future. The proband with the Q1224H mutation has another mutation in the *INS* gene promotor and is GAD+. Therefore, he will remain on insulin therapy for now but may incorporate sulfonylurea therapy in the future. In addition to causing diabetes, some mutations also causes DEND syndrome, which may be refractory to sulfonylurea therapy possibly because of poor penetration through the blood-brain-barrier.[38] For this reason, we also tested carbamazepine, another K_{ATP} channel inhibitor we recently uncovered,[30–32] and found that it also suppressed mutant channel activity. Of the five probands studied here, the proband with the R653Q mutation

was the only one with developmental delay in addition to NDM. However, because the R653Q proband is already in his 20s, intervention by carbamazepine may no longer be effective in reversing the neurological defects even if it does pass the blood–brain-barrier. As carbamazepine is already used for treating epilepsy and several other neurological disorders, it may be worth testing its efficacy towards the neurological defects in newly diagnosed DEND patients in the future.

In conclusion, this first functional characterization study from Indian NDM children carrying activating mutations in *ABCC8* offers insights into the mechanistic basis of genotype-phenotype relationships and helps deliver mechanism-based medicine in the clinical setting. Moreover, our novel finding that two L0 mutations have a profound effect on channel sensitivity to ATP inhibition, combined with recent high resolution structures of the channel, sheds new light on the importance of SUR1-L0 in ATP-induced Kir6.2 channel closure.

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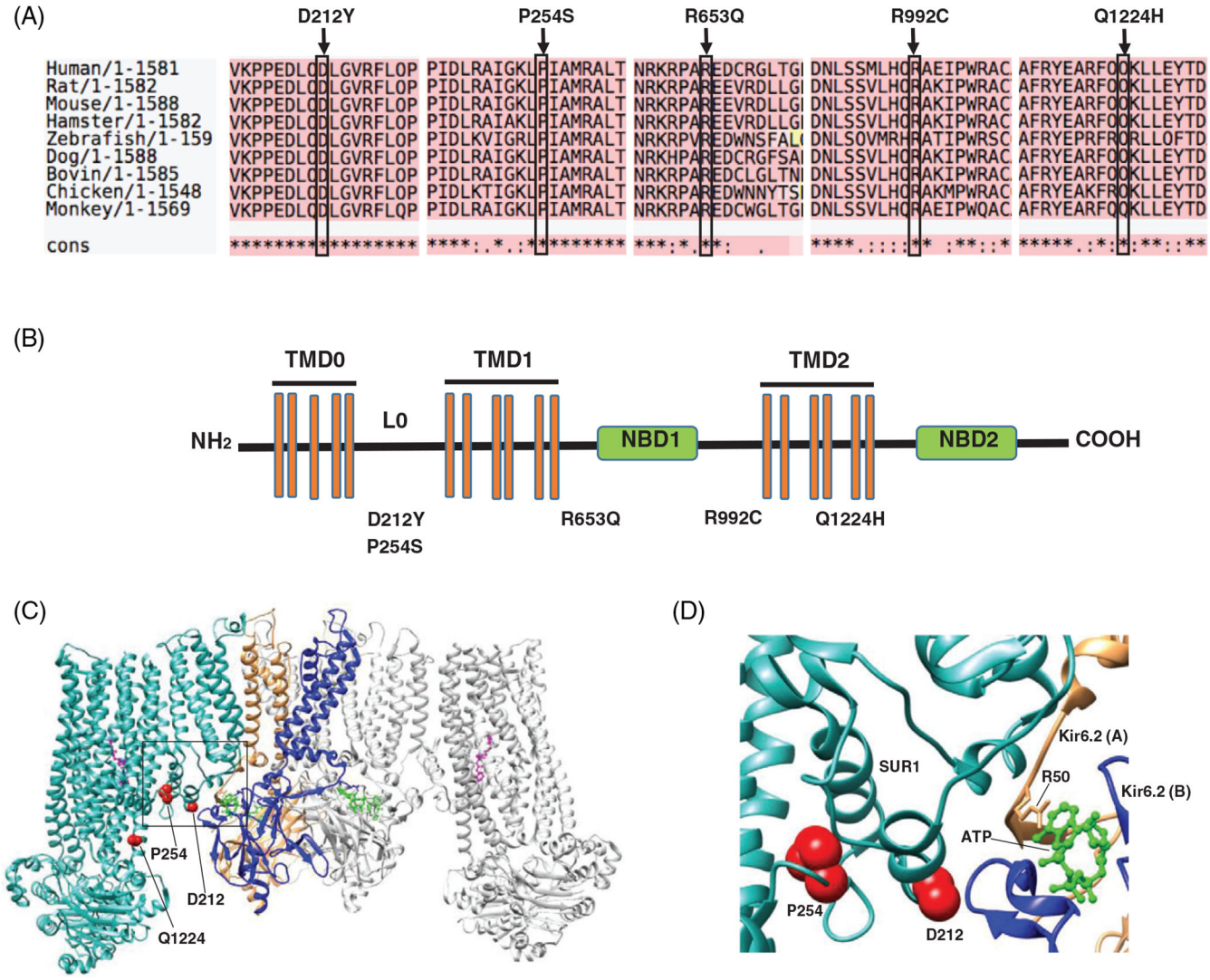

FIGURE 1.

Locations of sulfonylurea receptor 1 (SUR1) mutations. A, Multiple sequence alignment of SUR1 proteins from various species using ClustalW. Residues mutated are indicated by arrows and boxed to show conservation. B, Schematic representation of the SUR1 structure. Functional domains and mutation locations are marked. C, Three residues (D212, P254, and Q1224) that are mutated are mapped onto the recently published ATP-sensitive K^+ (K_{ATP}) channel structure bound to glibenclamide and ATP (PDB:6BAA). For clarity, the SUR1 subunits in the front and the back are removed. The three mutated residues are shown as red spheres in the SUR1 subunit colored in cyan. Two Kir6.2 subunits coordinating an ATP binding site close to the cyan SUR1 are colored in gold and blue. Glibenclamide and ATP are shown as magenta and green sticks, respectively. D, A close-up view of the boxed region in (C). D212 and P254 in the L0 of SUR1 are shown to illustrate their physical relationship with the ATP binding site formed by the two neighboring Kir6.2 subunits. For better orientation, the side-chain of a key ATP binding residue R50 in Kir6.2 subunit A (gold) is also shown

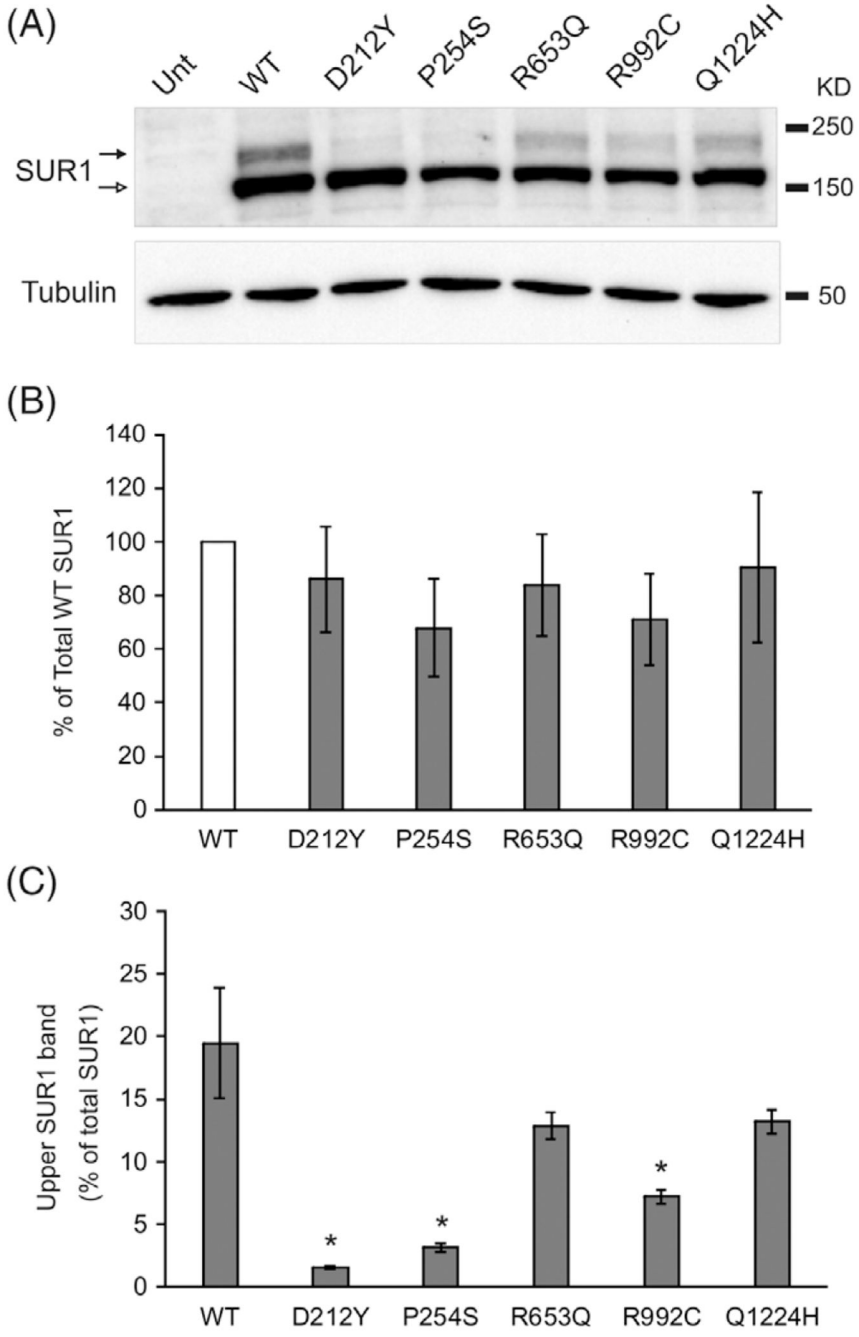

FIGURE 2.

Western blot analysis of wild-type (WT) or mutant sulfonylurea receptor 1 (SUR1) transiently co-expressed with Kir6.2 in COSm6 cells. A, A representative blot showing the steady state level of both the lower (gray arrow) and the upper band (black arrow) of mutant SUR1 compared to WT SUR1. Tubulin blot is shown below as a loading control. B, Quantification of the total SUR1 protein intensity (both lower and upper bands), expressed as % of total WT SUR1. C, Quantification of the upper SUR1 band, expressed as % of total SUR1 of each construct. In (B) and (C), each bar is the mean ± SEM of three blots. In (C),

statistical analysis was performed using one-way ANOVA with Dunnett's post hoc test. *$P <$ 0.01

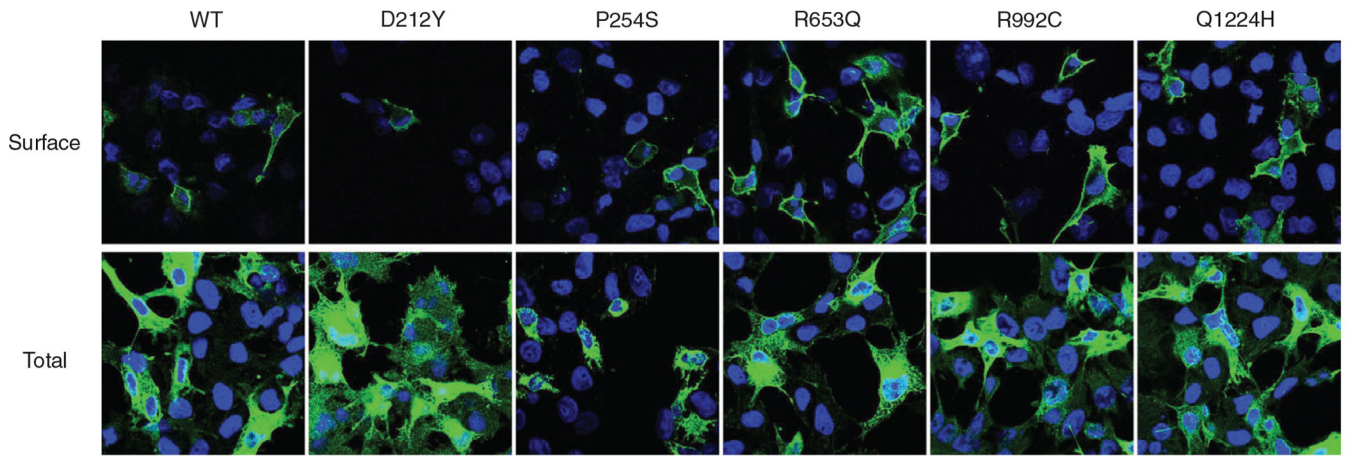

FIGURE 3.

Immunostaining of wild-type (WT) or mutant sulfonylurea receptor 1 (SUR1) co-expressed with Kir6.2 in COSm6 cells. *Top*: Surface staining of SUR1 using anti-FLAG primary antibody followed by goat anti-mouse Alexa488 secondary antibody as described in Section 2. Nuclei were stained with DAPI. *Bottom*: Staining of total cellular WT or mutant SUR1

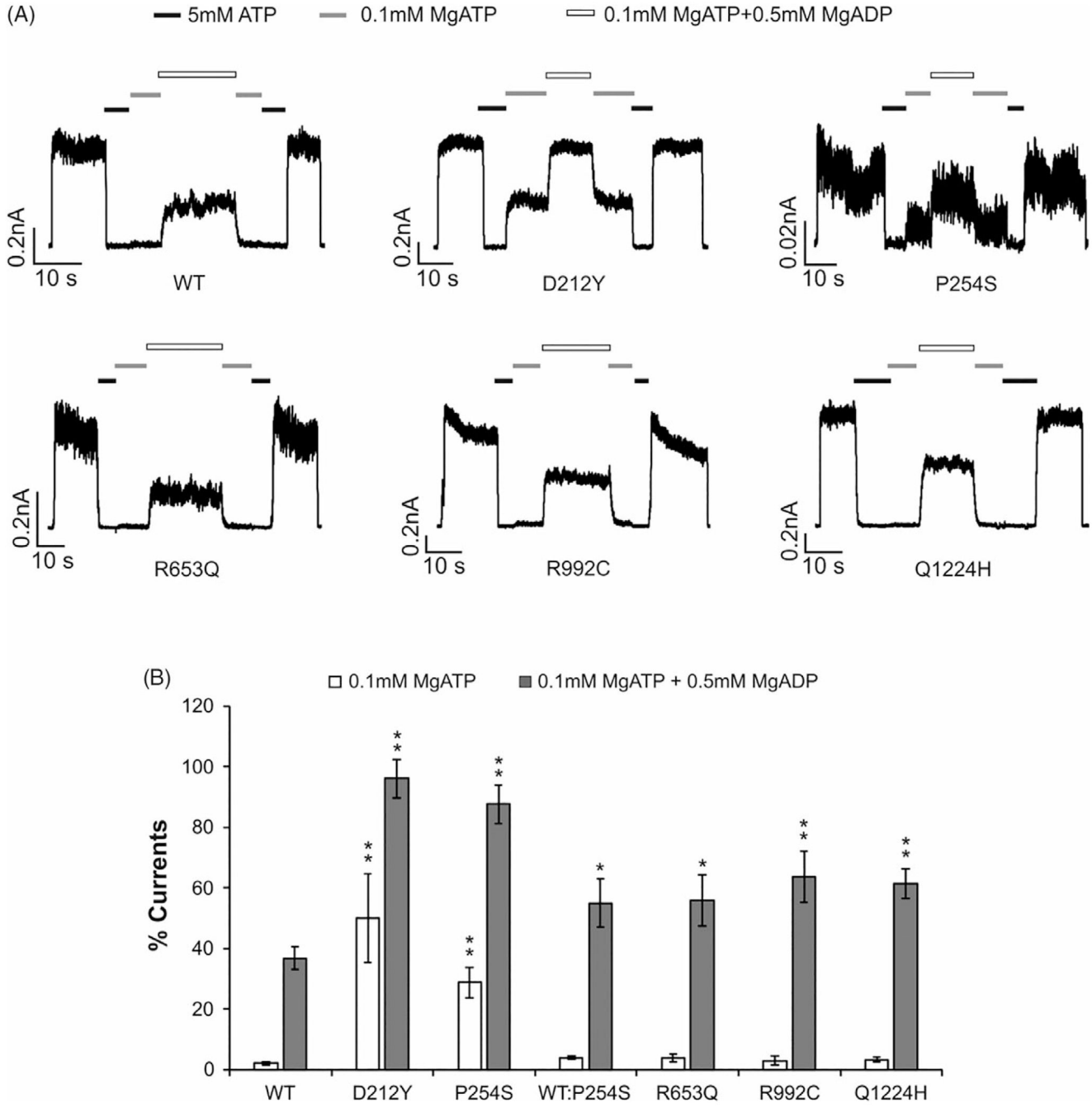

FIGURE 4.

Inside-out patch-clamp recording of wild-type (WT) and mutant channels in COSm6 cells. Mg-nucleotides response was monitored by exposing recombinant WT or mutant SUR1 channels to 5 mM ATP (to obtain baseline) or varying concentrations of ATP and ADP with 1 mM free Mg^{2+}. A, Representative recordings of WT and mutant channels. B, Bar graph shows % of currents relative to that seen in K-INT (140 mM KCl,10 mM K-HEPES,1 mM K-EGTA. pH 7.2) immediately after patch excision. Currents were measured at −50 mV in symmetrical K-INT solution, and inward currents are shown as upward deflections. All

SUR1 mutants as well as P254S expressed under simulated heterozygous condition with WT (WT:P254S) exhibited significantly higher activity compared to WT in 0.1 mM MgATP+0.5 mM MgADP. D212Y and P254S mutants also showed significantly increased currents in 0.1 mM MgATP compared to WT. Each bar represents mean ± SEM of 4 to 10 patches for mutants and 23 patches for the WT. *$P < 0.05$, **$P < 0.01$, comparison between the mutant and WT, by two-tailed, unpaired Student's *t* test

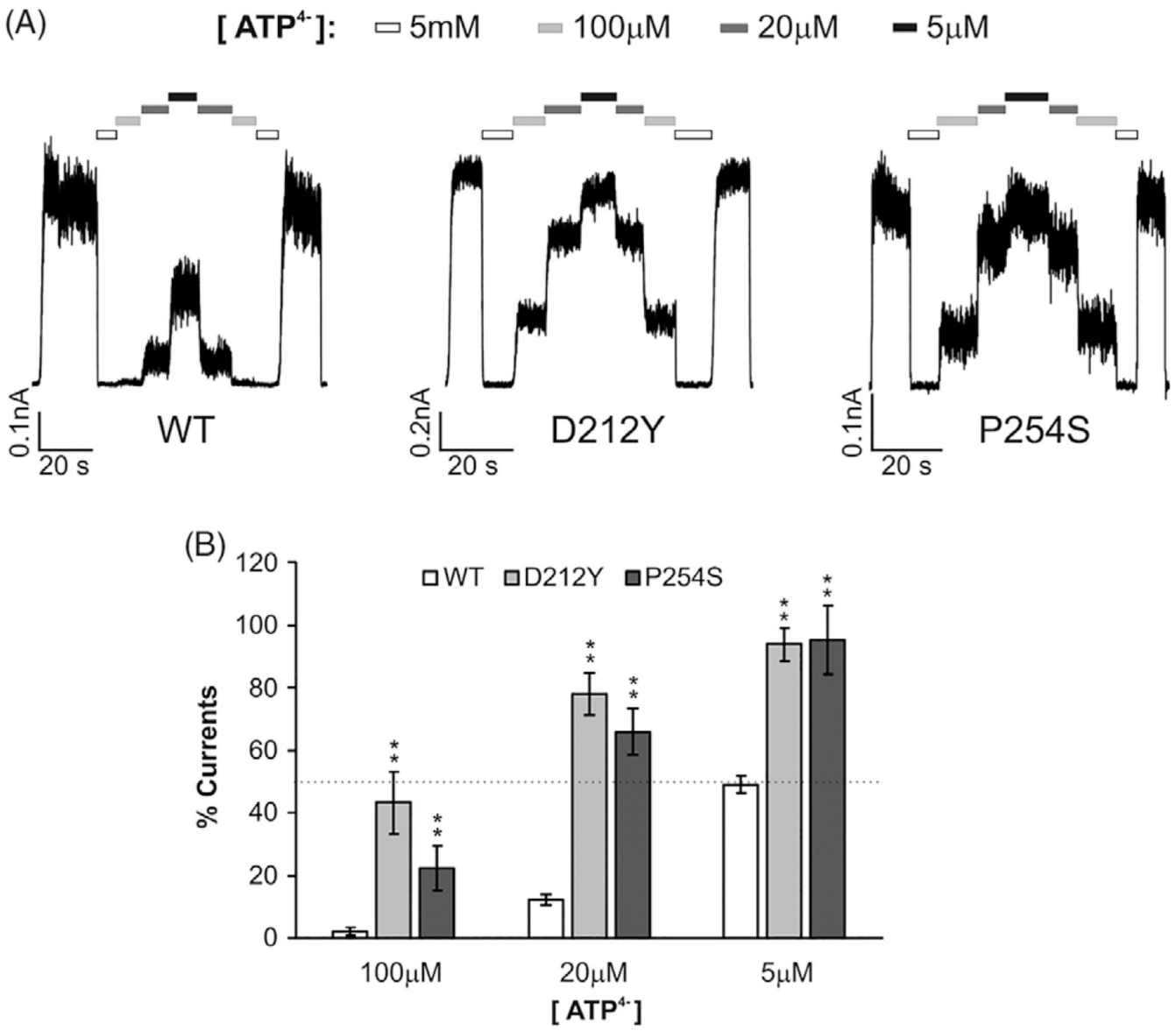

FIGURE 5.

Comparison of ATP^{4-} sensitivity in wild-type (WT) and the D212Y and P254S mutant channels. The activity of WT or mutant channels was monitored by exposing recombinant WT or mutant sulfonylurea receptor 1 (SUR1) channels to varying concentrations of ATP^{4-}. 1 mM ethylenediaminetetraacetic acid (EDTA) was included in the K-INT solution to chelate residual Mg^{2+} (K-INT/EDTA). 5 mM ATP was used to obtain baseline. A, Representative recordings of WT and mutant channels. B, Bar graph shows % of currents relative to that seen in K-INT/EDTA (140 mM KCl,10 mM K-HEPES,1 mM K-EGTA, 1 mM EDTA, pH 7.2). Currents were measured at −50 mV in symmetrical K-INT/EDTA solution, and inward currents are shown as upward deflections. The dashed lines indicate 50% currents. The D212Y and P254S mutants show significantly increased currents compared to WT in all three ATP concentrations tested. Each bar represents mean ± SEM

of 6-7 patches. $^{**}P < 0.01$, comparison between the mutant and WT by two-tailed, unpaired Student's *t* test

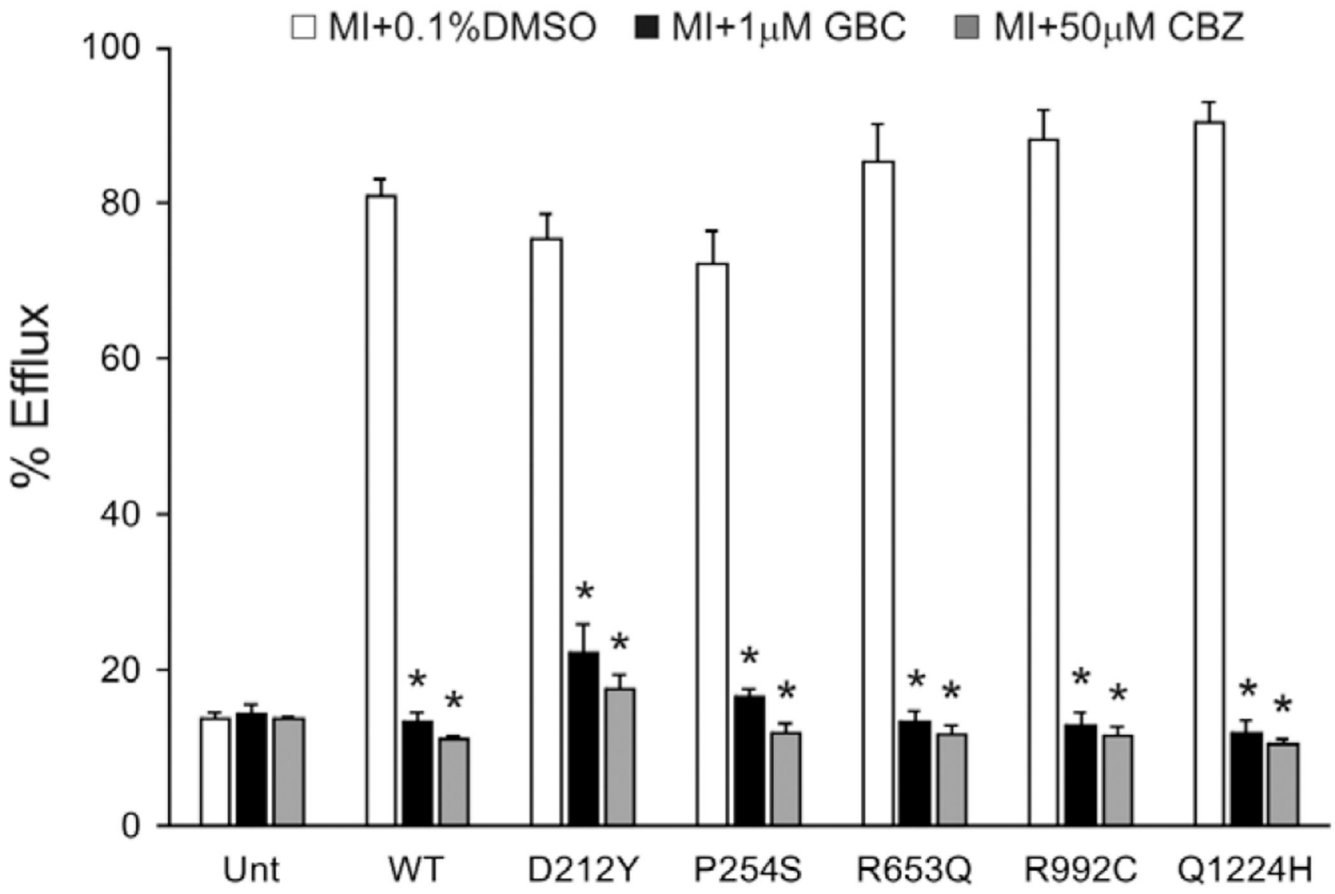

FIGURE 6.

$^{86}Rb^{+}$ efflux assay of COSm6 cells co-expressing Kir6.2 and wild-type (WT) or mutant sulfonylurea receptor 1 (SUR1). Channel response to glibenclamide (GBC) and carbamazepine (CBZ) was assessed in cells pretreated with metabolic inhibitors (1 mM 2-deoxy D-glucose and 2.5 μg/mL oligomycin) to activate channels. During efflux measurements, 0.1% dimethyl sulfoxide (DMSO) (vehicle control), 1 μM GBC or 50 μM CBZ were added to the solution as described in Section 2. Total efflux at the end of a 40-min period is shown and background efflux in untransfected cells is included as a control. Data represent mean ± SEM of three to four independent experiments. $*P < 0.01$, comparison between DMSO control and GBC- or CBZ-treated by two-tailed, unpaired Student's *t* test

TABLE 1

Clinical information of children carrying mutation in ABCC8 (NM_000352.4) with NDM phenotype

Clinical parameters	**p.D212Y[a] (c.634 g > t)**	**p.P254S[a] (c.760c > t)**	**p.R653Q[a] (c.1958 g > a)**	**p.R992C[a] (c.2974c > t)**	**p.Q1224H[a] (c.3672 g > t)**
Sex	Male	Male	Male	Male	Male
Age at onset (days)	60	90	96	75	90
Birth weight (kg)	2.5	2.8	3.2	2.5	2.5
Birth weight (centiles)	2	10	–	–	5
Random blood glucose(mg/dL)	235	570	774	527	450
HbA1C (%)	7.0	12.5	7.2	7.7	7.9
Developmental delay	No	No	Yes (delay in cognition and gross motor skills)	No	No
Other clinical features	Neonatal hepatitis, sepsis	–	–	–	–
Zygosity	Hetero	Homo	Hetero	Hetero	Hetero[b]
Clinical subtype	PNDM	PNDM	TNDM	PNDM	PNDM
Response to SU therapy	Yes	Not yet determined	Not yet determined	Yes	Not yet determined

Abbreviations: HbA1C, hemoglobin A1c; PNDM, permanent neonatal diabetes mellitus; TNDM, transient neonatal diabetes mellitus.

[a]According to gnomAD (http://gnomad.broadinstitute.org/), the population frequency for the p.P254S mutation is 0.00079% overall and 0.0032% in South Asian. For the p.R653Q mutation the total allele frequency is 0.052% and 0.44% in South Asian. The significantly higher allele frequency of these two mutations in South Asians compared to the general population suggests possible founder effects. The remaining three mutations (p.D212Y, p.R992C and p.Q1224H) are not found in the database.

[b]The proband carrying the Q1224H mutation also has another mutation in the *INS* (NM_0.000207.2) gene promoter (c.−176C > T).