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Cantú Syndrome Resulting from Activating Mutation in the *KCNJ8* Gene

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Abstract

ATP-sensitive potassium (K_{ATP}) channels, composed of inward-rectifying potassium channel subunits (Kir6.1 and Kir6.2, encoded by *KCNJ8* and *KCNJ11*, respectively) and regulatory sulfonylurea receptor (SUR1 and SUR2, encoded by *ABCC8* and *ABCC9*, respectively), couple metabolism to excitability in multiple tissues. Mutations in *ABCC9* cause Cantú syndrome, a distinct multi-organ disease, potentially via enhanced K_{ATP} channel activity. We screened *KCNJ8* in an *ABCC9* mutation-negative patient who also exhibited clinical hallmarks of Cantú syndrome (hypertrichosis, macrosomia, macrocephaly, coarse facial appearance, cardiomegaly, and skeletal abnormalities). We identified a *de novo* missense mutation encoding Kir6.1[p.Cys176Ser] in the patient. Kir6.1[p.Cys176Ser] channels exhibited markedly higher activity than wild-type channels, as a result of reduced ATP sensitivity, whether co-expressed with SUR1 or SUR2A subunits. Our results identify a novel causal gene in Cantú syndrome, but also demonstrate that the cardinal features of the disease result from gain of K_{ATP} channel function, not from Kir6-independent SUR2 function.

Keywords

KCNJ8; Kir6.1; Cantú syndrome; K_{ATP}; hypertrichosis

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Pore-forming proteins KCNJ8 (MIM# 600935) or KCNJ11 (MIM# 600937), commonly referred to as Kir6.1 or Kir6.2, respectively, generate ATP-sensitive potassium (K_{ATP}) channels in combination with regulatory subunits ABCC8 and ABCC9 (referred to as SUR1 and SUR2, respectively) (Nichols, 2006). SUR1 and Kir6.2 are expressed in pancreatic and neuronal tissues, and gain-of-function (GOF) mutations in either of these subunits give rise to neonatal diabetes, which is combined with neurological complications in the most extreme cases (Nichols, 2006). Heterozygous mutations in *ABCC9* have recently been shown to underlie 25 of 30 identified cases of Cantú syndrome (CS) (van Bon et al., 2012; Harakalova et al., 2012), a rare multi-organ disease first described by Cantú and colleagues (Cantu et al., 1982). The clinical hallmarks of CS (MIM# 239850) comprise congenital hypertrichosis, macrosomia at birth, macrocephaly, coarse facial appearance, cardiomegaly, skeletal abnormalities and developmental delay. In addition, heterozygous mutations in the *ABCC9* gene have been found in isolated cases of what has been termed hypertrichosis with acromegaloid facial appearance (HAFF) or acromegaloid facial appearance (AFA), characterized by some but not all of the cardinal features of CS (Czeschik et al., 2013). Some of these *ABCC9* (SUR2) mutations have been shown to generate K_{ATP} channels with reduced sensitivity to inhibitory ATP, *i.e.* a gain-of-function (Harakalova et al., 2012), when co-expressed with KCNJ11/Kir6.2 subunits. ABCC9 / SUR2 is expressed in many extra-pancreatic and extra-neuronal tissues, in overlapping distributions with Kir6.1 or Kir6.2 subunits. Since none of the CS features have been reported in patients with *KCNJ11* (Kir6.2) mutations, they are unlikely to arise from Kir6.2/SUR2 complexes. KCNJ8 (Kir6.1)/ ABCC9 (SUR2) complexes could be responsible for the disease phenotype, but multiple spliced forms of ABCC9 are known to exist, and may exist in sub-cellular compartments and function without partner Kir6.1 or Kir6.2 subunits (Aggarwal et al., 2010; Stoller et al., 2010). Thus, it remains unclear exactly how the features of CS result from ABCC9 mutations.

The clinical phenotype of the proband at the age of 3 months was originally described by Engels *et al* (Engels et al., 2002). Following the realization that this CS patient harbored a distinct molecular basis, we have now carried out a detailed re-evaluation of the patient at the age of 13 years. The patient shows key clinical hallmarks of CS, including congenital hypertrichosis, macrosomia at birth, macrocephaly, coarse facial appearance, cardiomegaly, skeletal abnormalities and developmental delay (Supp. Table S1). At the time of re-evaluation he also had excessive gingival hyperplasia (Fig. 1B). Echocardiography at 13 years of age showed normal biventricular function with signs of non-compaction of the left ventricular apical myocardium, sonography of parenchymal abdominal organs was within the normal range, as were ECG and 24 hour blood pressure measurements. The patient's weight was 41 kg (25.–50. centile), height was 144 cm (10.–25. centile) and BMI was 19.5 (+0.3 SDS). He appeared disproportionate with a sitting height of 76 cm, arm span of 151.5 cm (10–25th centile) and arm span: height ratio >97th centile. His chest x-ray showed the same broadening of ribs as initially reported (Engels et al., 2002). X-ray of his left hand showed his bone age to be 10½ years at 13 years of age, corresponding to a delay of 2½ years. Laboratory studies showed essentially undetectable serum IGF-1 levels (< 25 ng/ml, Ref. value 131–690) and a markedly decreased IGFBP3 value of 1.4 μg/ml (Ref. value 2.6 – 8.9). Arginine tolerance and clonidine test revealed absolute growth hormone (GH)

deficiency (basal and all stimulated GH concentrations below 1.0 ng/ml). GHRH testing resulted in subnormal GH secretion (max. GH 2.8 ng/ml).

Comparison of the present case with recently published CS patients carrying *ABCC9* mutations (Supp. Table S1) (Harakalova et al., 2012; van Bon et al., 2012) shows that the present patient exhibits all clinical hallmarks of CS, as well as 10/12 CS associated facial/cranial features; 2/5 cardiac features; 7/17 skeletal abnormalities, visible on radiographic studies; and 3/15 additional previously reported CS-associated features.

To date, mutations in *ABCC9* are the only known genetic cause of CS (Czeschik et al., 2013; Harakalova et al., 2012; van Bon et al., 2012). In our initial study we found 9 of 12 patients diagnosed with CS to carry missense mutations in *ABCC9* (Harakalova et al., 2012; van Bon et al., 2012). We performed Sanger sequencing of the three coding exons of *KCNJ8* in the 3 unexplained patients, including the present case. *KCNJ8* was chosen as the most promising candidate gene because of the functional considerations stated above. The study was approved by the local ethics committees, and all participating patients gave written informed consent. Parental consent was given on behalf of patients younger than 18 years of age; the study was explained to children capable of giving assent, and they provided oral assent. In the proband we identified a missense mutation Chr12(GRCh37):g.21919406A>T (NM_004982.2:c.526T>A), in the *KCNJ8* gene, encoding a missense mutation (p.Cys176Ser) in the Kir6.1 protein (Fig. 1). We did not observe the mutation in any of 2,096 in-house exomes, nor is it reported in any of 6,503 individuals from the exome variant server (Exome Variant Server, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/) [March 2014]). The variant has been deposited in the LOVD 3.0 (http://databases.lovd.nl/). It is predicted as ‘deleterious’ by SIFT (score 0.03), and probably damaging by PolyPhen2 (score 1.0). The affected amino acid is fully conserved in both mammalian Kir6.1 and Kir6.2 proteins suggesting a key role in channel function. Paternity was proven and sample mix-up excluded by STR marker analysis. The mutation was not present in maternal or paternal DNA and hence was presumed to have arisen *de novo*. Primer sequences and PCR conditions are available upon request.

To assess the effect of the p.Cys176Ser mutation on channel activity, mutant and wild type *KCNJ8* (Kir6.1) cDNAs were co-transfected with *ABCC8* (SUR1) or *ABCC9* (SUR2A) cDNAs. Channel activity was assessed under normal metabolic conditions, in metabolically inhibited conditions (mimicking tissue ischemia), and in the presence of pharmacological potassium channel openers (KCOs), using ^{86}Rb efflux assays (see Methods). We also examined the channel activity resulting from Kir6.1[pSer422Leu], a mutation reported in several studies to be associated with J-wave abnormalities in the electrocardiogram (Barajas-Martinez et al., 2011; Medeiros-Domingo et al., 2010). As shown in Fig. 1 and Supp. Figure S1, very similar fluxes were measured for WT Kir6.1 and p.Ser422Leu channels (see below). Measurable basal conductance was present for WT Kir6.1 with SUR1, but only MI- or KCO-stimulated fluxes were present for WT plus SUR2A. In contrast, significant fluxes were present for Kir6.1[p.Cys176Ser] with SUR1, and both MI- and KCO-stimulated fluxes were higher for each Kir6.1[p.Cys176Ser]/SUR combination. These experiments establish that the p.Cys176Ser mutation indeed causes a gain-of-function in the Kir6.1 channel,

leading to markedly enhanced channel activity even under basal metabolic conditions, in co-expression with either SUR1 or SUR2A subunits.

To gain further insight to the mechanism by which the mutation enhances channel activity, we turned to patch-clamp experiments. The majority of K_{ATP} channels in vivo are probably formed as homomeric Kir6.1 tetramers or Kir6.2 tetramers, although there is evidence that heteromeric Kir6.1/Kir6.2 combinations may also be present in cardiac (Bao et al., 2011) and smooth (Flagg et al., 2010; Insuk et al., 2003) muscle, for instance. Since the original cloning and expression of K_{ATP} subunit genes, it has been clear that Kir6.1 channels are experimentally considerably more labile in excised patches than are Kir6.2 channels, and channel activity rapidly runs down in the absence of Mg-nucleotides, complicating the assessment of both inhibitory nucleotide sensitivity and intrinsic open probability of the channel. In the patient, the mutation is present in only one allele and since the active channels are tetramers, expressed K_{ATP} channels in native cells will therefore be expected to consist of a mixture of both p.Cys176Ser and wild type Kir6.1 or Kir6.2 subunits. To take advantage of this fact and additionally test the effect of heterozygosity, we co-expressed a 1:1 mixture of wild-type or mutant Kir6.1 cDNAs with wild type Kir6.2 and SUR2A cDNAs (Fig. 2). These mixtures generated measurable fluxes in tracer studies and stable currents in excised patches. From these measurements it is evident that enhanced channel activity in intact cells expressing p.Cys176Ser (Fig. 2A and Supp. Figure S2) result from reduced ATP sensitivity (Fig. 2B,C). We cannot formally rule out the possibility that altered trafficking and increased plasma membrane channel density may also play a role, but such effects have not previously been described for mutations in Kir channel pores. The findings indicate that the heterozygous p.Cys176Ser mutation in Kir6.1 will overactivate any native K_{ATP} channels in which it is present. The results are very consistent with the effects of the exactly equivalent, and well-studied mutation, p.Cys166Ser in Kir6.2, which reduces ATP sensitivity of expressed channels by ~50-fold (Loussouarn et al., 2000; Tucker et al., 1998). Several additional mutations at this residue in Kir6.2 which also cause marked gain-of-function have been identified as causal in the most severe form of neonatal diabetes, associated with developmental delay and epilepsy (Gloyn et al., 2006).

Cantú syndrome (CS) was first characterized as such by J.M. Cantú in 1982 (Cantu et al., 1982). A genetic cause was recently reported (Harakalova et al., 2012; van Bon et al., 2012), with coding mutations identified in the *ABCC9* gene, in 25 of 30 patients. The present study reveals that a heterozygous mutation in *KCNJ8*, encoding the Kir6.1 pore-forming subunit of K_{ATP} channels, can also cause CS. A recent study identified a different mutation in *KCNJ8* (encoding p.Val65Met) in another CS patient (Brownstein et al., 2013), although there was no functional characterization of mutant channels. The marked gain-of-function in expressed Kir6.1[p.Cys176Ser] channels (Figs. 1,2), which mirrors the well-studied properties of Kir6.2 channels with mutations at the equivalent p.Cys166 residue (Gloyn et al., 2006; Loussouarn et al., 2000; Tucker et al., 1998), clearly establishes p.Cys176Ser as a severe gain-of-function. Thus gain-of-function mutations in both *ABCC9* and *KCNJ8* cause essentially the same hallmark CS features in the affected patients (Supp. Table S1), indicating that these features all result from gain-of-function of K_{ATP} channels formed from these subunits. This is a subtle but critical conclusion: previous studies (Aggarwal et al.,

2010; Stoller et al., 2010) have raised the possibility of Kir6-independent roles of SUR2 proteins and, in the absence of the present findings, some or all of the features of CS could conceivably have arisen from non-channel functions of the SUR2 protein. The results underscore the key role of K_{ATP} channels in coupling cell membrane potential with diverse tissue functions. Our results are consistent with the finding that vascular smooth muscle contractility is markedly decreased in the presence of K_{ATP} channel openers (Flagg et al., 2010) and in transgenic mice expressing mutant Kir6.1 subunits with reduced sensitivity to inhibitory ATP (Li et al., 2013), potentially explaining some of the key findings in CS, including patent ductus arteriosus, as was also present in the patient reported here.

A novel finding is the puzzling combination of biochemical signs of absolute growth hormone (GH) deficiency, yet postnatal growth with height within the normal range. Pituitary GH secretion is mainly regulated by two hypothalamic neuropeptides. GHRH stimulates GH release, whereas somatostatin inhibits GH secretion (Muller et al., 1999). In addition, a number of GH-releasing peptides are able to modulate GH release. The rat pituitary expresses several Kir channel alpha-subunits, including Kir6.1 (Wulfsen et al., 2000). GHRP-2, one member of the GH-releasing neuropeptides, exerts its GH secretory effect through a reduction in potassium current (Xu et al., 2002). It is therefore tempting to speculate that GOF mutations in pituitary K_{ATP} channels exert an opposite effect on GHRH stimulation, leading to resistance to hypothalamic induction of GH release. On the other hand, GH insufficiency yet normal growth can only be explained by a concurrent stimulation of long bone growth.

A common K_{ATP} pathway can account for direct overlap of CS features resulting from mutations in *ABCC9* and *KCNJ8*, as well as with features of overexposure to minoxidil and other K_{ATP} channel openers (Avatapalle et al., 2012; Kaler et al., 1987; Nguyen and Marks, 2003). However, this does not provide an obvious explanation for many CS features. For instance, macrocephaly and characteristic facial features, as well as skeletal abnormalities are present in both *ABCC9* and *KCNJ8* patients, but while K_{ATP} channels generated by these subunits have been reported in human chondrocytes (Rufino et al., 2013) and osteoblasts (Kawase et al., 1996), their role in maturation and proliferation remains unknown, and the cellular pathway involved is not obvious. Interestingly, minoxidil has been reported to induce pseudoacromegalic features in the absence of elevated GH or IGF-1 (Nguyen and Marks, 2003). Similarly, epicanthal folds and deep plantar creases might result from K_{ATP} gain-of-function, but the underlying cause is unknown. Finally, pericardial effusion, polydramnios and lymphoedemia, potentially all related problems of membrane barrier breakdown, are unexplained.

Finally, we would note that several other studies have reported a different mutation (p.Ser422Leu) in the Kir6.1 protein to be associated with ‘early repolarization syndrome’ (ERS), characterized by abnormalities in the J-point of the electrocardiogram (Haissaguerre et al., 2009). Recent studies have reported that the mutation enhances channel activity (Medeiros-Domingo et al., 2010), by reducing ATP-sensitivity (Barajas-Martinez et al., 2011). If so, there is a clear inconsistency: neither J-wave abnormalities nor other arrhythmias have been reported in CS patients, and none of the CS features have yet been reported for ERS patients. Our own data (Fig. 1C) indicate that, in recombinant COS cells,

this variant does not affect Kir6.1-dependent K_{ATP} channel activity, consistent with the recent recognition that p.Ser422Leu may actually represent a common variant (~4%) in Ashkenazim subjects, and not clearly associated with ERS (Veeramah et al., 2013).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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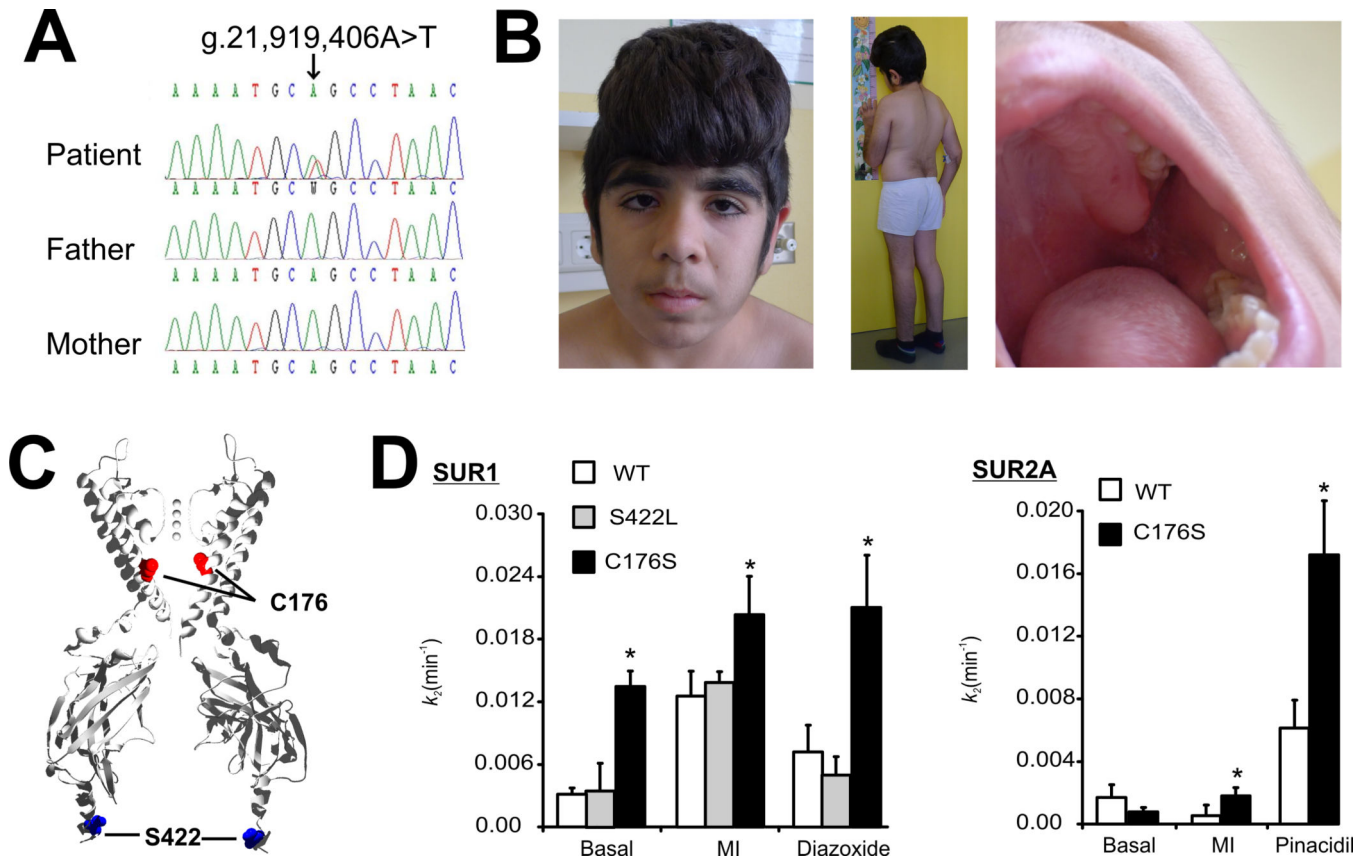

Fig 1. Gain-of-function *KCNJ8* mutation Kir6.1[p.Cys176Ser] underlies Cantu Syndrome

(A) Sequence chromatogram of patient and unaffected parents genomic DNA confirms de novo g.21,919,406A>T transition mutation in the patient, that is absent in the mother and father. (B) Clinical phenotype of the patient. Photographs of the patient at 13 years reveal extensive hypertrichosis, macrocephaly, coarse facial appearance, long arm and torso to height ratio, and gingival hyperplasia with thickened lips. (C) Ribbon diagram of two of the four Kir6.1 subunits that form the K^+-selective pore in K_{ATP}, based on the crystal structures of KirBac1.1 (Kuo et al., 2003) and cytoplasmic domain of Kir3.1 (Nishida et al., 2002). Shown are Kir6.1 residues mutated in CS (p.Cys176Ser), and reported in association with the J-wave syndrome (p.Ser422Leu). (D) Rate constants for K_{ATP}-dependent $^{86}Rb^+$ efflux (k_2) in basal conditions relative to metabolic inhibition (MI) for WT and mutant K_{ATP} channels (mean ± s.e.m., from 4–6 experiments). *P < 0.01 compared to wild-type K_{ATP} by unpaired Student's *t* test.

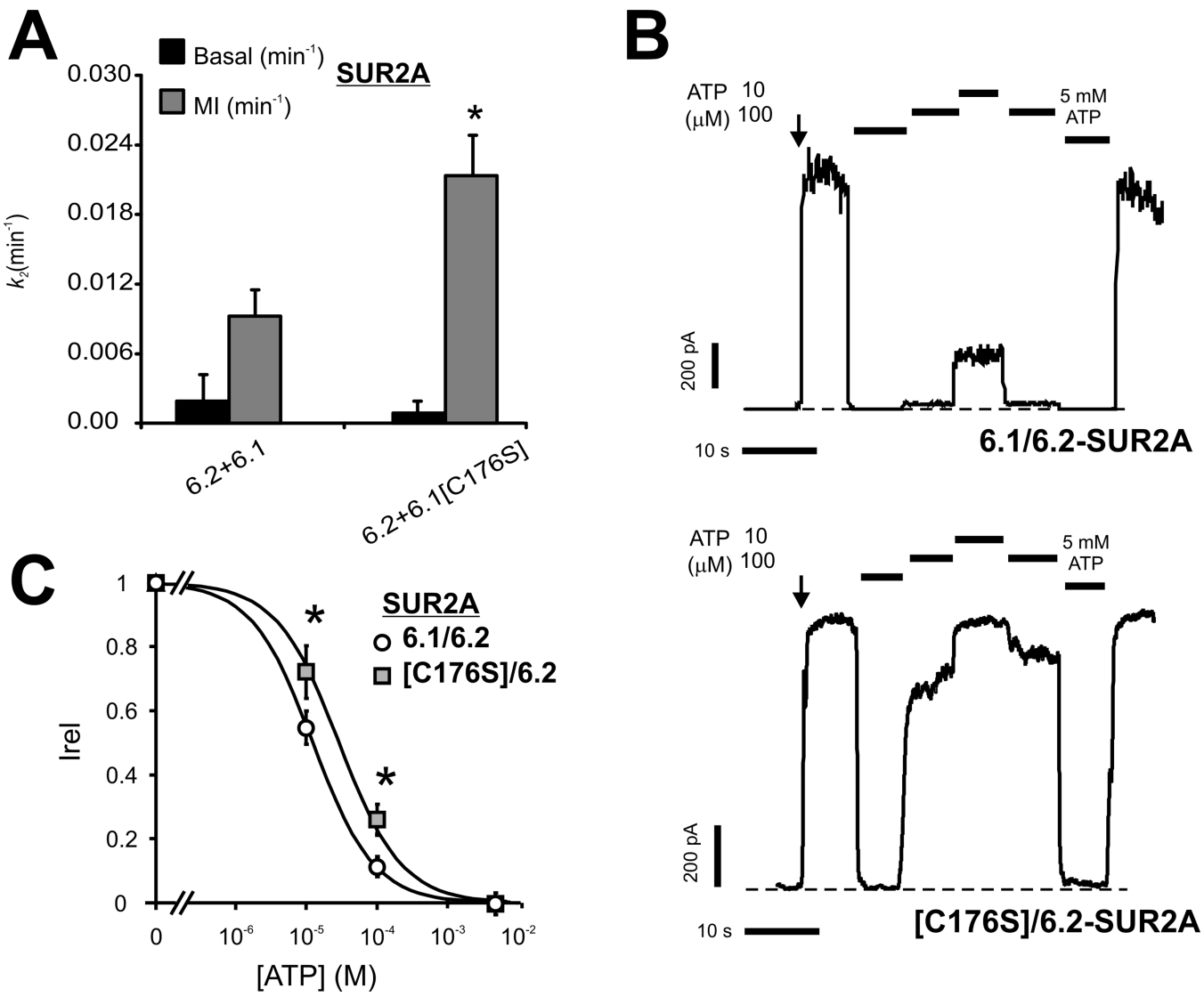

Fig. 2. Reduced ATP-sensitivity of reconstituted heteromeric K_{ATP} channels containing Kir6.1[p.Cys176Ser] subunits

(A) Rate constants for K_{ATP}-dependent $^{86}Rb^{+}$ efflux (k_2) in basal conditions relative to metabolic inhibition (MI) from COS cells expressing heteromeric Kir6.1/Kir6.2 or Kir6.1[p.Cys176Ser] (C176S)/Kir6.2 plus SUR2A K_{ATP} channels. (B) Representative currents recorded from inside-out membrane patches from COS cells expressing heteromeric Kir6.1/Kir6.2 or Kir6.1[p.Cys176Ser] (C176S)/Kir6.2 plus SUR2A K_{ATP} channels at −50 mV in K_{int} solution (see methods). Patches were exposed to differing [ATP] and baseline current was determined by exposure to ATP (5mM). (C) Steady-state dependence of membrane current on [ATP] (relative to current in zero ATP (I_{rel})) for wild-type and p.Cys176Ser-containing channels. Data points represent the mean ± s.e.m. (n = 6–8 patches). The fitted lines correspond to least squares fits of a Hill equation (see methods). *P < 0.01 compared to wild-type K_{ATP} by unpaired Student's t test.