

In Vitro Recovery of ATP-Sensitive Potassium Channels in β -Cells From Patients With Congenital Hyperinsulinism of Infancy

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OBJECTIVE—Congenital hyperinsulinism in infancy (CHI) is characterized by unregulated insulin secretion from pancreatic β -cells; severe forms are associated with defects in *ABCC8* and *KCNJ11* genes encoding sulfonylurea receptor 1 (SUR1) and Kir6.2 subunits, which form ATP-sensitive K⁺ (K_{ATP}) channels in β -cells. Diazoxide therapy often fails in the treatment of CHI and may be a result of reduced cell surface expression of K_{ATP} channels. We hypothesized that conditions known to facilitate trafficking of cystic fibrosis transmembrane regulator (CFTR) and other proteins in recombinant expression systems might increase surface expression of K_{ATP} channels in native CHI β -cells.

RESEARCH DESIGN AND METHODS—Tissue was isolated during pancreatectomy from eight patients with CHI and from adult cadaver organ donors. Patients were screened for mutations in *ABCC8* and *KCNJ11*. Isolated β -cells were maintained at 37°C or 25°C and in the presence of 1) phorbol myristic acid, forskolin and 3-isobutyl-1-methylxanthine, 2) BPDZ 154, or 3) 4-phenylbutyrate. Surface expression of functional channels was assessed by patch-clamp electrophysiology.

RESULTS—Mutations in *ABCC8* were detected for all patients tested ($n = 7/8$) and included three novel mutations. In five of eight patients, no changes in K_{ATP} channel activity were observed under different cell culture conditions. However, in three patients, in vitro recovery of functional K_{ATP} channels occurred. Here, we report the first cases of recovery of defective K_{ATP} channels in human β -cells using modified cell culture conditions.

CONCLUSIONS—Our study establishes the principle that chemical modification of K_{ATP} channel subunit trafficking could be of benefit for the future treatment of CHI. *Diabetes* 60:1223–1228, 2011

Congenital hyperinsulinism in infancy (CHI) is characterized by severe hypoglycemia, which manifests in the neonatal period. The disease may be limited to a localized region of the pancreas (focal CHI) as a result of somatic loss of maternal alleles and expression of paternal mutations or may be diffuse and inherited with Mendelian genetics (1). The most severe forms of CHI are caused by loss-of-function mutations in the genes encoding the subunits of the ATP-sensitive K⁺ (K_{ATP}) channel: *ABCC8* (encoding sulfonylurea receptor 1 [SUR1]) and *KCNJ11* (encoding Kir6.2); both genes are located on chromosome 11p15 (1,2). In β -cells, these channels are complexes consisting of four SUR1 and four Kir6.2 subunits, which assemble in the endoplasmic reticulum (ER) and are glycosylated and modified as they pass through the *cis*-, *medial*-, and *trans*-golgi network before being expressed at the cell surface. Although studies of β -cells from patients with CHI have proved the link between *ABCC8* and *KCNJ11* gene defects and loss-of-function of K_{ATP} channels (3,4), recombinant techniques have been used to further understand the mechanisms of this loss. Disease-causing mutations engineered in rodent SUR1 and Kir6.2 have been expressed in mammalian and nonmammalian expression systems (e.g., COSm6 cell line, *Xenopus* oocytes) and found to cause incorrect assembly of the channel complex, impaired trafficking from the ER, or loss of nucleotide regulation (4–8). These experiments also demonstrated the importance of specific amino acid motifs present on both SUR1 and Kir6.2 for anterograde and retrograde trafficking of K_{ATP} channels (reviewed in [1,9]). Similar approaches have been used to demonstrate that some CHI-related defects can be overcome by altering the cell culture environment (6,10,11). However, to date no studies have examined methods to recover defective K_{ATP} channels in native tissue, which could be of relevance in the future treatment of CHI. We now report for the first time rescue of K_{ATP} channels in patient β -cells using chemical mediators, kinase activators, and reduced temperature.

RESEARCH DESIGN AND METHODS

Tissue was isolated (with permission) from cadaver human organ donors and from eight patients with CHI who required subtotal pancreatectomy for intractable hypoglycemia. Table 1 summarizes patient details. Islets of Langerhans were isolated as previously described (3,12). Total RNA was extracted from islets and cells using TRIzol reagents (Invitrogen, Paisley, U.K.) and subjected to RT-PCR using primers designed and tested in-house. All PCR reactions consisted of an initial denaturation step of 94°C for 5 min followed by 35 cycles of 94°C for 1 min, Ta°C for 1 min, and 72°C for 1 min followed by

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TABLE 1
CHI patient tissue details

Patient (#)	Age at surgery	Histology	Gene defect	Genotype	Reference
1	10 weeks	Diffuse	Presumed Homozygous, <i>ABCC8</i>	Unknown	—
2	12 weeks	Diffuse	Homozygous, <i>ABCC8</i>	c.1467+5G>A	Novel mutation
3	12 weeks	Diffuse	Compound Heterozygous, <i>ABCC8</i>	p.Arg998X/p.Ser1449dup	25
4	12 weeks	Diffuse	Homozygous, <i>ABCC8</i>	c.3992-9G>A	3
5	7 weeks	Diffuse	Homozygous, <i>ABCC8</i>	c.3992-9G>A	3
6	3.5 years	Diffuse	Compound Heterozygous, <i>ABCC8</i>	p.Gly70Glu/p.Arg1419Gly	3
7	12 months	Diffuse	Compound Heterozygous, <i>ABCC8</i>	p.Lys242fs/p.Arg1437X	Novel mutations
8	4 weeks	Focal	Paternal uniparental isodisomy, <i>ABCC8</i>	p.Arg598X	3

Seven patients were found to have diffuse CHI, and one patient was defined as focal CHI. Consent for genotyping was obtained from seven patients, and all were found to carry defects in the *ABCC8* gene. Islet mRNA was isolated from patient #1, and RT-PCR suggested that CHI was caused by defects in *ABCC8* expression and not *KCNJ11*. Where CHI causing mutations have been previously described, see references indicated.

a final elongation stage of 72°C for 10 min. For detection of mRNA encoding *KCNJ11* (Genbank Accession number NM_000525), primer sequences were as follows: (F) ACA AGA ACA TCC GGG AGC, (R) ACA CGT AGC ATG AAG CAG AGG with Ta 60°C. For detection of three different regions of *ABCC8* (Genbank Accession number AF087138), primer sequences were as follows: (F) AGA CTG CCC ACA AGA AGC (bases 748–765), (R) AGA AGA AAA ACC ACA TGA (bases 1335–1317) with Ta 58°C; (F) GAC CCA CAA GCT ACA GTA CC (bases 2693–2712), (R) CAC TCC ACA GTG ACA GAC G (bases 3295–3276) with Ta 58°C; (F) TCT CGA ATA CAC AGA CTC C (bases 3713–3731), (R) ACA GTG TGC TAT CTG AGC (bases 4386–4368) with Ta 60°C. PCR products were resolved on 1.2% agarose gel prepared with Tris-borate EDTA buffer (Fisher Scientific, Loughborough, U.K.) containing 1.2 µg/mL ethidium bromide (Promega, Madison, WI). Bands were visualized and photographed under ultraviolet light.

Groups of dispersed islet cells were cultured at either 37°C or 25°C in a humidified atmosphere of 5% CO₂/air mixture for a minimum of 16 h with or without addition of 10 µmol/L BPDZ 154 (B. Pirotte, Universite de Liege, Belgium), 100 µmol/L diazoxide (Sigma, Poole, U.K.), or 2.5 mmol/L 4-phenylbutyrate (4-PB) (Triple Crown America, Perkasie, PA). Alternatively, cells were incubated for 1 h before experimentation at 37°C with 100 µmol/L 3-isobutyl-1-methylxanthine (IBMX), 2 µmol/L forskolin, and 10 nmol/L phorbol myristic acid (PMA; Sigma, Poole, U.K.). All data were obtained using the cell-attached or inside-out recording configurations of the patch-clamp technique as previously described (3,12). In all experiments, the integrity of the recording configuration was assessed by inducing activation of the high conductance Ca²⁺- and voltage-gated K⁺ channel by perfusing the inside face of the cell membrane with a Ca²⁺-containing solution (3,12). To select for K_{ATP} channel currents, the pipette contained a standard NaCl-rich bathing solution containing (in mol/L) 140 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.13 MgCl₂, 10 HEPES, 2.5 glucose (pH 7.4 with NaOH), and the bath solution contained (in mol/L) 140 KCl, 10 NaCl, 1.13 MgCl₂, 1 EGTA, 2.5 glucose, and 10 HEPES (pH 7.2 with KOH) for all recordings. Reagents were added at the concentrations indicated in the text. Diazoxide and BPDZ 154 were added to working solutions from a stock solution dissolved in DMSO (Sigma) giving a final concentration of less than 0.1% vol/vol DMSO. Statistical comparisons were made using Student *t* tests or ANOVA with values of *P* < 0.05 taken to be significant. All data are expressed as mean values ± SEM.

RESULTS

In this study, insulin-secreting cells were investigated from eight patients with CHI who underwent pancreatectomy following failure to respond to medical treatment. Permission for genotype analysis was obtained for seven patients (#2–#8) and all were found to carry defects in *ABCC8* (Table 1; Fig. 2A). Genotyping was declined for patient #1; therefore RNA was isolated from dispersed islets and RT-PCR analysis for three regions of *ABCC8*, and *KCNJ11* carried out. Primers directed to discrete regions of the N-terminus of *ABCC8* and for *KCNJ11* amplified appropriate products, but failed to do so when primers were directed toward the center or COOH-terminal regions of *ABCC8* mRNA.

In isolated inside-out patches of membrane from control human β-cells, K_{ATP} channels were spontaneously open and

activated in the presence of ATP by ADP (500 µmol/L) and diazoxide (200 µmol/L) (Fig. 1A). In all eight CHI cases, we found that SUR1 defects were linked to a functional loss of K_{ATP} channels in β-cells since the average peak current varied between patients from zero to approximately 10% (*n* = 109 recordings) of the control values obtained from human adult β-cells (*n* = 189). These data are similar to our previously reported findings (3,4,12). Figure 1A (*center panel*) shows typical recordings of β-cells from patient #1 when the cells were maintained under standard cell culture conditions (i.e., at 37°C without additional supplementation of the tissue culture medium). In contrast with control recordings, no operational or normally regulated channels were recorded in either intact cells (*n* = 8/8) or isolated inside-out patches (*n* = 8/8; Fig. 1A). However, when β-cells from the patient were maintained at 25°C for a minimum of 16 h, K_{ATP} channels were restored (*n* = 7/7) and were found to be responsive to ADP and diazoxide (Fig. 1A and B). This modification did not affect the activity of K_{ATP} channels in control human β-cells (Fig. 1C). Following incubation at 25°C with 2.5 mmol/L 4-PB, no further improvements were observed for patient #1 β-cells. In contrast, we did see an increase in K_{ATP} channel activity in *ABCC8* (*Arg998X/Ser1449dup*) β-cells (patient #3) maintained at 37°C with 4-PB. This procedure led to the appearance of K_{ATP} channel currents, which were increased by 4.4 ± 2-fold in 3/4 cells compared with control values, but was not associated with the restoration of nucleotide-dependent activation of channels (*n* = 3). Due to limited tissue availability, we did not test effects of incubation at 25°C in patient #3 β-cells.

A similar pattern involving recovery of functional K_{ATP} channel activity was found in *ABCC8* (*c.1467+5G>A*) β-cells (patient #2), first when exposed to a combination of IBMX (100 µmol/L), PMA (10 nmol/L), and forskolin (2 µmol/L) for 1 h at 37°C immediately before the experiment (*n* = 4/4; Fig. 1D) and second when cells were maintained at 37°C for 24–48 h with BPDZ 154 (10 µmol/L) added to the cell culture medium (*n* = 6/6; Fig. 1D). BPDZ 154-induced recovery of channel activity was also seen in *ABCC8* (*Arg998X/Ser1449dup*) β-cells (patient #3; *n* = 4 recordings, 4.3 ± 1-fold increase in activity), but this was not associated with 0.5 mmol/L ATP-induced inhibition of channels (*n* = 4).

DISCUSSION

In healthy β-cells, glucose-induced closure of K_{ATP} channels is a key regulator of stimulus-secretion coupling. The

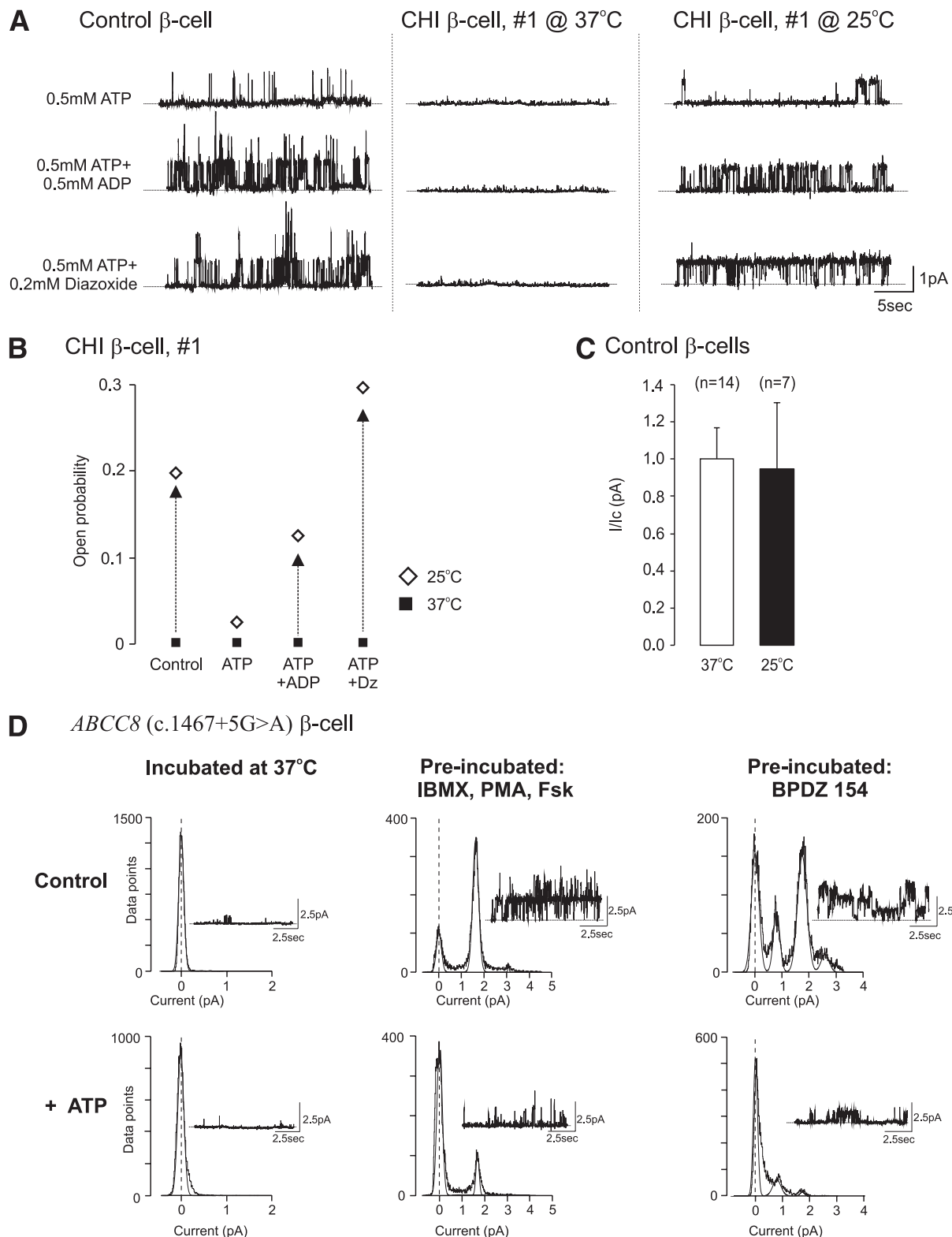


FIG. 1. In vitro recovery of K_{ATP} channels in CHI β -cells. **A:** Data from control (adult) human β -cells to illustrate the modulation of ATP-inhibited channels (open probability [P_o] = 0.03 ± 0.02 , $n = 11$ cells), by ADP (0.5 mol/L, $P_o = 0.1 \pm 0.03$, $n = 9$) and diazoxide (0.2 mol/L, $P_o = 0.3 \pm 0.05$, $n = 5$) and from CHI β -cells maintained at either 37°C (standard conditions) or at 25°C. Note the marked increase in channel activity in CHI β -cells maintained at low temperature and how ADP and diazoxide induce an increase in the activity of channels in the presence of ATP (0.5 mol/L). **B:** The effects of cells maintained at 25°C on channel open probability. In CHI β -cells, no K_{ATP} channels were recorded under standard conditions (open probability = 0, ■) but were readily observed in cells maintained at the lower temperature (◇). **C:** Maintaining control β -cells at 25°C had no effect on the average magnitude of K_{ATP} channels in isolated patches ($n = 7/7$). **D:** The recovery of K_{ATP} channels in *ABCC8* (c.1467+5G>A) β -cells following short-term exposure to IBMX (0.1 mmol/L), forskolin (Fsk; 2 μ mol/L), and PMA (10 nmol/L) for 1 h or long-term exposure to BPDZ 154 (10 μ mol/L). Representative single-channel current data are shown alongside amplitude histogram profiles following Gaussian fitting of the data (smoothed lines). Note how in control β -cells >99% of the events occur at 0 pA (indicated by the dotted line) consistent with the absence of functional channels, but that following treatment open events are apparent, which are sensitive to ATP (0.5 mmol/L).

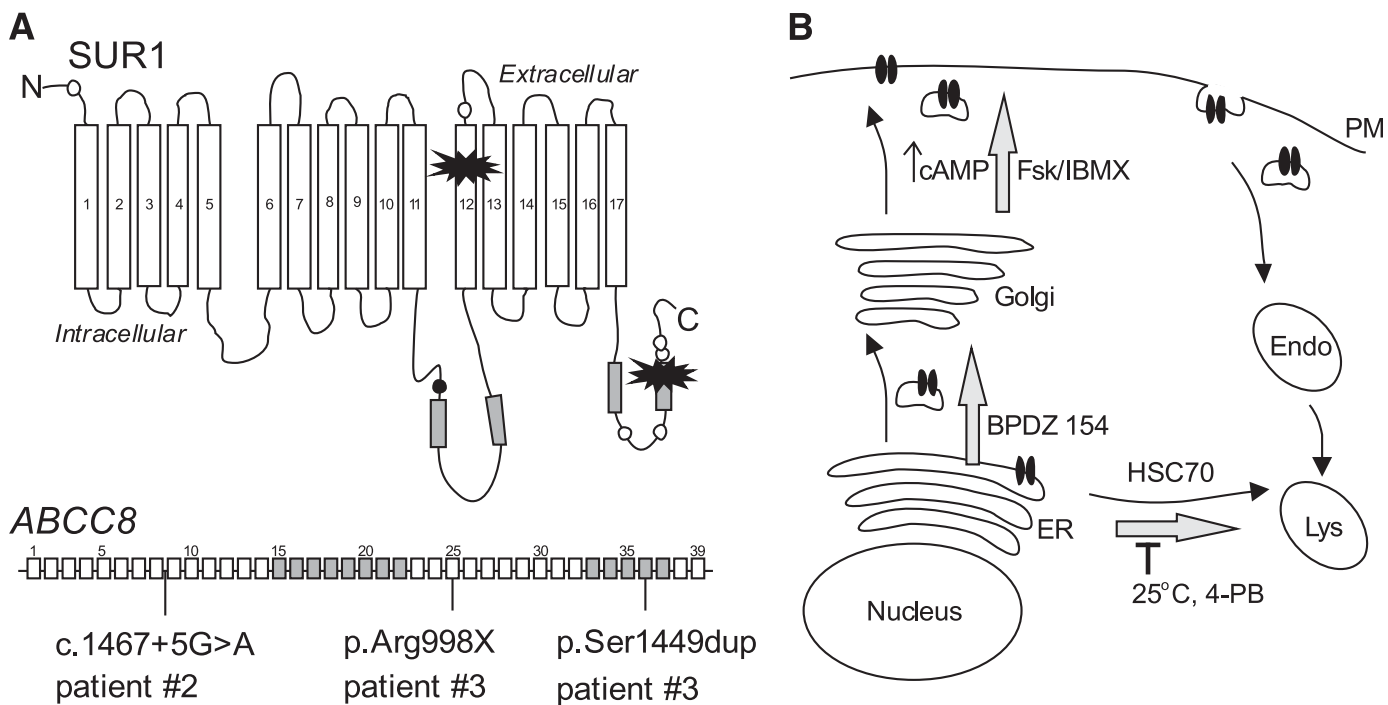


FIG. 2. K_{ATP} channel structure and trafficking in β -cells. **A:** A consensus model of the structure of SUR1 (*top*) with predicted transmembrane domains (numbered 1–17), extracellular NH₂ terminus, and intracellular COOH terminus. Shaded rectangles represent the intracellular nucleotide binding domains. Predicted sites of mutations for patient #3 are indicated (black stars); note that the intronic mutation c.1467+5G>5 does not have a predicted site on SUR1. \circ , known anterograde (forward) trafficking motifs on SUR1; \bullet , the RKR retention motif, which must be masked by correct channel assembly to permit forward trafficking. The intron/exon structure of *ABCC8* is shown *below* with shaded rectangles marking the exons predicted to encode the nucleotide binding domains. **B:** The trafficking of K_{ATP} channel proteins (black ellipses) in β -cells. Gray arrows indicate suggested mechanisms of compounds and conditions described in this study to increase forward trafficking of K_{ATP} channels. PM, plasma membrane; Endo, endosome; Lys, lysosome.

pivotal role of these channels in this process is central to our understanding of how loss-of-function mutations in *ABCC8* or *KCNJ11* cause CHI (1), whereas gain-of-function mutations lead to neonatal diabetes (2). Gene defects that render K_{ATP} channels less sensitive to ATP prevent correct ATP:ADP sensing and can cause impaired insulin secretion and diabetes (2). By contrast, decreased K_{ATP} channel density and/or loss of ADP sensitivity is associated with inappropriate electrical activity and uncontrolled insulin release leading to CHI (3). Here we report recovery of K_{ATP} channel activity in β -cells from patients with CHI following incubation with chemical mediators, kinase activators, and reduced temperature to increase trafficking of the channels to the cell membrane. In the field of cystic fibrosis (CF) it has long been recognized that enhancement of cell surface trafficking of the defective ATP-binding cassette (ABC) protein cystic fibrosis transmembrane regulator (CFTR) may be of therapeutic value (13). Most CF patients (90%) carry a deletion of phenylalanine at position 508 (Δ F508) of *ABCC7*, which encodes CFTR (14). This mutation results in misfolding of the protein leading to ER retention where it is subsequently polyubiquitinated and targeted for degradation (15). Incubation of cells engineered to express Δ F508 CFTR at 25°C was found to result in recovery of CFTR activity by stabilizing protein folding in the ER (16). We found that low temperature incubation also led to recovery of mutant K_{ATP} channel activity in CHI patient tissue. By analogy with studies of the processing of mutant CFTR (16), we believe that low temperature incubation of CHI β -cells stabilized K_{ATP} channel subunits during folding and assembly in the ER, and thereby allowed the protein to escape the ER

quality control measures. During the biogenesis of K_{ATP} channels, it has recently been shown that SUR1 can interact with heat shock cognate (HSC)70, heat shock protein (HSP)90, and HSP40 (17). We speculate that the success of low temperature incubation arises through disruption of mutant K_{ATP} channel degradation involving HSC70, and this results in increased functional expression of the channel at the cell membrane (Fig. 2B). We explored this potential mechanism further using 4-PB, which increases the trafficking of mutant CFTR via down-regulation of HSC70 expression (18). Similarly, in our studies 4-PB also led to an increase in K_{ATP} channel activity providing further support to the role of HSC70 in degradation of mutant K_{ATP} channels (17).

In a separate set of experiments, we found that incubation of CHI β -cells with a kinase activating cocktail also led to K_{ATP} channel recovery. This effect could be due to signaling pathways associated with activation of protein kinase C, or the downstream effects of increased cytosolic cAMP, such as activation of protein kinase A or other cAMP-dependent signaling pathways including cAMP kinase, A-kinase anchor proteins, cAMP regulatory element-binding protein (CREB), and cAMP-regulated guanine nucleotide exchange factors (cAMP-GEFs). Modulation of kinase activity has previously been reported to increase trafficking of other ion channels and transporters such as the bile salt export pump (also a member of the ABC protein family; 19), aquaporin-2 (20), and CFTR (21). It has also been reported that PKC activation is associated with a reduction in surface K_{ATP} channel expression as a result of a reduction in recycling of endocytic vesicles and the diversion of K_{ATP} channel-containing vesicles to lysosomal

degradation (22). The overall positive effect of kinase activation in CHI β -cells suggests that activation of cAMP-dependent kinases or similar mechanisms may have led to increased expression or function of K_{ATP} channels (Fig. 2B). Further studies are required to characterize the precise signaling pathways involved.

Although diazoxide has previously been reported to increase trafficking of K_{ATP} channels in recombinant systems (6), the effects on trafficking of the related compound BPDZ 154, a more potent K_{ATP} channel agonist (12), have not been investigated previously. In this study we found that BPDZ 154 caused recovery of K_{ATP} channels in CHI β -cells suggesting that the compound, like diazoxide, may influence channel trafficking in addition to opening K_{ATP} channels. In recombinant cells several channel modulators have now been reported to act as “chemical chaperones” by mediating the recovery of defective channels (6,10), including diabetes-causing mutations (11). Although the mechanisms responsible for these actions have not been fully investigated, the agents are thought to stabilize biogenesis of the channel complex rather than the SUR1 subunit and thereby facilitate ER exit of the complex. A similar action may also explain the long-term effects of BPDZ 154 on CHI β -cells (Fig. 2B).

Our series of experiments also included five cases of CHI in which either short- or long-term modification of the cell culture conditions failed to evoke any increase in the expression or regulation of K_{ATP} channels in CHI β -cells ($n = 51$ experiments). This is not surprising since *ABCC8* (or *KCNJ11*) defects will alter the protein in many different ways and not all will have configurations that enable the protein to be potentially liberated from the ER. Indeed, in recombinant studies several *ABCC8* mutations have been shown to be resistant to chemical chaperones (5,8,10).

Little is known about the effects of splice site mutations in *ABCC8* on the subsequent expression of SUR1 protein. However, our results demonstrate that the presence of splice-site mutations does not necessarily lead to the absolute loss of channel protein since patients #4 and #5 demonstrated recordable (but defective) K_{ATP} channel activity in untreated cells. We have no information on the effects of the mutations reported in our study on transcription and translation efficiency of the *ABCC8* gene and cannot therefore rule out that the culture conditions we used somehow modified or stabilized these processes, leading to apparent K_{ATP} channel recovery in some of our experiments, including patient #2 β -cells expressing a splice site mutation.

In summary, we have demonstrated rescue of K_{ATP} channel activity for the first time in pancreatic β -cells isolated from patients with congenital hyperinsulinism. Clinically, enhancement of mutant K_{ATP} channel trafficking to the cell membrane of β -cells could be beneficial for the treatment of CHI, especially if these channels retained regulatory properties or could be activated by K_{ATP} channel agonists. With the introduction of rapid genetic testing in CHI, pharmacogenomics is becoming increasingly realistic for tailoring drug treatment to individuals. Therefore it is possible that once the gene mutations causing CHI are identified, optimization of trafficking conditions for each patient may permit successful enhancement of K_{ATP} channel activity in some cases. By analogy with CF where large scale screening efforts for small molecule “correctors” of CFTR trafficking have been successful (23), this could eventually have an impact on clinical treatment of

CHI. Indeed, several pharmacological modulators of trafficking pathways are currently undergoing clinical trials for the treatment of other diseases (24).

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P.D.P., C.B.-C., and S.E.F. researched and provided data. S.E., R.R., and K.H. contributed to data analysis and discussion. M.S. researched and provided data. P.C. and P.d.L. contributed to data analysis and discussion. M.J.D. contributed to data analysis and discussion and wrote the manuscript. K.E.C. researched and provided data, contributed to data analysis and discussion, and wrote the manuscript.

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