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Abstract

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Reference

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Severe Congenital Hyperinsulinism due to a Mutation in the Kir6.2 Subunit of the K_{ATP} Channel Impairing Trafficking and Function

Abbreviated title: Congenital hyperinsulinism and K_{ATP} channel

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Abbreviations: K_{ATP} , ATP sensitive K^+ channel; CHI, Congenital Hyperinsulinism; ER, endoplasmic reticulum; TIRF, Total internal reflection fluorescence; Tolbu, Tolbutamide; Diazo, Diazoxide; PHHI, Persistent Hyperinsulinemic Hypoglycemia of Infancy.

Abstract

Context: The ATP-sensitive potassium (K_{ATP}) channel, assembled from the inwardly rectifying potassium channel Kir6.2 and the sulfonylurea receptor 1 (SUR1), regulates insulin secretion in beta cells. A loss of function of K_{ATP} channels causes depolarization of beta cells and congenital hyperinsulinism (CHI), a disease presenting with severe hypoglycemia in the newborn period.

Objective: Identification of a novel mutation in Kir6.2 in a patient with CHI. Molecular and cell-biological analysis of the impact of this mutation.

Design and Setting: Combining immunohistochemistry, advanced live fluorescence imaging and electrophysiology in HEK293T cells transiently transfected with mutant Kir6.2.

Patient and Intervention: The patient presented with macrosomia at birth and severe hyperinsulinemic hypoglycemia. Despite medical treatment, the newborn continued to suffer from severe hypoglycemic episodes and at four months of age subtotal pancreatectomy was performed.

Main Outcome Measure: Patch-clamp recordings and confocal microscopy in HEK293T cells.

Results: We have identified a homozygous missense mutation, H259R, in the Kir6.2 subunit of a patient with severe CHI. Co-expression of Kir6.2_{H259R} with SUR1 in HEK293T cells completely abolished K_{ATP} currents in electrophysiological recordings. Double immunofluorescence staining revealed that mutant Kir6.2 was partly retained in the endoplasmic reticulum (ER) causing decreased surface expression as observed with total internal reflection fluorescence (TIRF). Mutation of an ER-retention signal partially rescued the trafficking defect without restoring whole cell currents.

Conclusion: The H259R mutation of the Kir6.2 subunit results in a channel that is partially retained in the ER and non-functional upon arrival at the plasma membrane.

Introduction

K_{ATP} channels couple the metabolism of a cell to its electrical activity and are widely expressed. In the heart these channels are involved in ischemic preconditioning (1) while in the brain they have neuro-protective roles during ischemia (2). In the pancreas K_{ATP} channels are localized at the plasma membrane of beta cells and to the insulin secretory granule (3), where they sense ATP, which is tightly regulated by glucose levels. Thus, an increase in glucose concentration leads to a higher ATP to ADP ratio and to K_{ATP} closure, beta cell depolarization and insulin secretion. The functional K_{ATP} channel is an octameric complex formed by four SUR1 and four inwardly Kir6.2 subunits (4). Chronically impaired channel function causes depolarization of the beta cell with sustained insulin secretion, a condition known as congenital hyperinsulinism (CHI), previously termed as persistent hyperinsulinemic hypoglycemia of infancy (PHHI). Histologically, two different forms of CHI exist, a diffuse form, where all the islets of the pancreas are altered and a focal form with a small region of hyperplastic beta cells, surrounded by normal pancreatic islets (5). Overall, 40-65% of patients with CHI present a focal form (6-8). A majority of studies have identified mutations in the SUR1 subunit associated with CHI (9, 10). To date, more than ten mutations have been identified with CHI (11-16). One Kir6.2 mutation (Y12X) caused the synthesis of a truncated non-functional protein (12), while an other mutation (W91R) showed defective channel assembly with a rapid degradation in the ER (17).

Here we identified a new homozygous mutation in the Kir6.2 subunit in a patient with severe CHI. Combining immunohistochemistry, advanced live fluorescence imaging and electrophysiology, we demonstrate that the H259R mutation leads to a non-functional K_{ATP} channel and impaired trafficking to the cell membrane.

Materials and Methods

Genetic analysis

Genomic DNA was extracted from peripheral blood using the Genomic PrepBlood DNA Isolation Kit (Amersham Pharmacia Biotech, Piscataway, NJ) after informed consent has been obtained. Individual exons of the *ABCC8* gene coding for SUR1 (39 exons) and the *KCNJ11* gene coding for Kir6.2 (1 exon) (GenBankTM accession numbers *ABCC8*: L78207, *KCNJ11*: NM_000525) were amplified by PCR and screened for mutations by direct nucleotide sequencing (18).

Molecular Biology

The plasmid pECE-hKir6.2 (WT) (a generous gift from Dr. J. Bryan) was used to generate the pECE-hKir6.2 (H259R) using the *in vitro* QuikChangeTM Site-Directed mutagenesis kit (Stratagene, The Netherlands) according to the manufacturer's protocol. Following primers were used: forward, 5'-CCGCTGATCATCTACCGTGTCATTGATGCCAACAGC-3' and reverse, 5'-GCTGTTGGCATCAATGACACGGTAGATGATCAGCGG-3'. The mutation was

confirmed by DNA sequencing. Wild type and mutant *KCNJ11* cDNA were subcloned into the pCDNA3 vector (Invitrogen, Basel, Switzerland). The pCDNA3-hKir6.2(WT) and the pCDNA3-hKir6.2(H259R) were used to generate pcDNA3-hKir6.2_{AAA}(WT) and the pCDNA3-hKir6.2_{AAA}(H259R) with the *in vitro* QuikChangeTM Site-Directed mutagenesis kit by using the following primers: forward, 5'-CCGCGGGCCCCTGGCCGCGCCAGCGTGCCCATGG-3' and reverse, 5'-CCATGGGCACGCTGGCCGCGCCAGGGGCCCGCGG -3'. All the mutations were confirmed by DNA sequencing. Human *ABCC8* cDNA (pECE-hSUR1 (a generous gift from Dr. J. Bryan, Baylor College of Medicine, Houston) was subcloned into the pCDNA3 vector.

Cell culture and transfections

The human embryonic kidney (HEK293T) cell line was grown and maintained in RPMI 1640 (Seromed, Basel, Switzerland), supplemented with 5% fetal calf serum, 5% newborn calf serum (Life Technologies, Basel, Switzerland), 100Units/ml penicillin (Seromed, Basel, Switzerland), 100 µg/ml streptomycin (Seromed, Basel, Switzerland) and 2mM glutamine (Life Technologies, Basel, Switzerland). HEK293T cells were transiently co-transfected using the calcium phosphate precipitation technique with human *ABCC8* and *KCNJ11* cDNA (WT, WT_{AAA}, H259R, H259R_{AAA}) in a 4:1 ratio (1 µg of *ABCC8* and 0.25 µg of *KCNJ11*) (19). After 48 hours, cells were used for the patch clamp technique, for immunohistochemistry or TIRF microscopy experiments. We transfected HEK293T cells with two GFP fusion constructs (Kitl-EGFP (Mb-EGFP) and KLS-EGFP (ER-EGFP)) to either label the cell membrane or the ER, using the same conditions as described previously, to validate our TIRF experiments (20).

Electrophysiological Measurements

As described previously (21), all experiments were performed at room temperature (20-22°C), the pipette solution consisted of 10 mM NaCl, 140 mM KCl, 1 mM MgCl₂, 10 mM Hepes, 1 mM EGTA, 1 mM MgATP (pH 7.2 with KOH), the extracellular solution used was 145 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM Hepes, 10 mM D-glucose (pH 7.2 with NaOH) and the equilibrium potential for K⁺ ions (E_{K^+}) was -82 mV. Membrane slope conductance values (G_m) were calculated from dI/dV , using ramp voltage-clamp protocol (between -120 and -40 mV, a voltage range symmetrical to E_K). dI was determined from tolbutamide-sensitive currents.

Immunohistochemistry

HEK293T cells were stained with the following antibodies: goat anti-Kir6.2 (sc11228, Santa Cruz Biotechnology, Heidelberg, Germany), guinea pig anti Kir6.2 (generous gift from Dr. B. Schwappach), goat anti-calreticulin (generous gift from Dr. M. Michalak) to mark the ER, mouse anti-giantin (generous gift from Dr. H. Hauri H. and Dr. M. Spiess) to stain the Golgi apparatus. Following secondary antibodies were used: anti-goat FITC, anti-mouse Alexa568 and anti-goat alexa 568 (Molecular Probes, Leiden, The Netherlands). For colocalization experiments of the protein Kir6.2 with the plasmic membrane marker, toxin-GPI-alexa546 (generous gift from Dr. F. van der Goot) was used. Slides were viewed on a Zeiss LSM 510 confocal microscope (Carl Zeiss AG, Göttingen, Germany).

Total internal reflection fluorescence (TIRF) microscopy

We used an inverted microscope Axiovert 100M (Carl Zeiss AG, Göttingen, Germany) equipped with a high numerical aperture objective (100x NA 1.45; Carl Zeiss AG) and a combined epi-fluorescence/TIRF adapter (TILL photonics, Gräfelfing, Germany). Fluorophores were excited at 488nm with a 150 mW argon-ion laser through a monomode optical fiber (488/568/647nm) and the fluorescence filter set containing a laser clean-up filter (488/10), dichroic mirror (DCLP500) and band pass emission filter (BP525/50). Images were acquired with a 12-bit CCD camera (Orca 9742-95; Hamamatsu, Japan). The laser shutter, the camera and the microscope set up were controlled by the Openlab software (Improvision, Basel, Switzerland).

Quantification and Statistical analysis

The percentage of co-localization of the protein Kir6.2 (WT or H259R) with the different markers used in this study was calculated with the Metamorph Software version 6.2r4 (Universal Imaging, Puchheim, Germany). Results are expressed as mean +/- SD. Where indicated, the statistical significance of the differences between groups was estimated by the Mann-Whitney Test or the t-test. Statistical significance is indicated with * for $p < 0.01$, ** for $p < 0.001$.

Results

Case synopsis

The infant was born at term after an uneventful pregnancy and presented with macrosomia (body weight 4460 g, body length 54 cm, both values > P90). 30 minutes after birth, severe hypoglycemic episodes were observed (glucose level of 31 mg/dl (1.7 mmol/l) with a simultaneous insulin level of 172 mU/l) leading to the diagnosis of hyperinsulinism. The newborn was treated with i.v. glucose at a rate of 20 mg/kg/min and diazoxide was added at a dose of 17 mg/kg/d. Despite treatment, the newborn continued to suffer from severe hypoglycemic episodes and octreotide (17 µg/kg/d) and nifedipine (0.25 mg/kg/d) were added successively without therapeutic success. Continuous i.v. glucagon (1 mg/d) was needed to stabilize the blood glucose levels. The patient developed clinical signs of cardiac insufficiency; cardiac ultrasound showed biventricular hypertrophy. At four months of age a pancreatic catheterization with measurements of insulin levels (6), suggested a diffuse form of CHI. At five months of age, a subtotal pancreatectomy (95%) was performed and pancreatic histopathology confirmed the diagnosis. After pancreatectomy the infant became diabetic and was treated with an insulin pump. At the age of 20 months the total daily insulin dose was 0.54U/kg with a HbA1c of 6.9%. Blood glucose measurements showed maximal levels of 181 mg/dl (10 mmol/l). The insulin dose was gradually tapered and eventually stopped at the age of 23 months. Two months after the insulin treatment was completely stopped the HbA1c was at 6.4% and daily blood glucose measurements varied between 74.5 mg/dl (4.1 mmol/l) and 145 mg/dl (8 mmol/l). The HbA1c values further decreased to 5.4% at the age of 36 months without any treatment and no hypoglycemic episodes were encountered.

Genetic analysis

Sequencing of the 39 exons of the *ABCC8* gene, encoding the SUR1 protein, revealed no mutation. In contrast, a homozygous missense mutation, 776A>G, was found in the *KCNJ11* gene, encoding the Kir6.2 protein, leading to a change in the amino acid sequence (H259R). The mutation was located close to the C-terminal end at a highly conserved site, found in 52 proteins related to Kir6.2. This makes DNA sequence polymorphism therefore unlikely (Fig. 1). Both parents were found to be heterozygous for the 776A>G mutation.

Functional analysis of the mutant K_{ATP} channel

To study the functional impact of the mutation in the Kir6.2 protein on the K_{ATP} channel, we used the patch clamp technique in the whole cell configuration. HEK293T cells were transiently co-transfected with mutant *KCNJ11* cDNA and wild type *ABCC8* cDNA. GFP cDNA was added to identify successfully transfected cells. As shown in Fig. 2, K_{ATP} currents were absent in cells expressing the mutant K_{ATP} channel (panel B), but present in the wild type (panel A). Earlier studies have reported that functional recovery of K_{ATP} currents in the case of mutation in the SUR1 subunits can be obtained with diazoxide (22). As shown in Fig. 2B (middle panel), diazoxide had no effect on the H259R mutant K_{ATP} channel. In fact the current was completely absent in all cells with the mutated K_{ATP} channel ($n = 8$, $p < 0.001$, Fig. 2B and C).

Retention of mutant K_{ATP} channel in the endoplasmic reticulum (ER)

The absence of current could result from several abnormalities such as decreased protein synthesis, defects in assembly and trafficking, increased degradation (17) or impaired function of the channel itself (23). In our case, the H259R mutation did not appear to interfere with protein synthesis, since Kir6.2 protein could be synthesized in vitro (data not shown). It has been shown that K_{ATP} channels are subjected to quality control during ER trafficking, whereby the correct assembly of the subunits masks retention signals (24, 25) and allows membrane insertion. To test for trafficking defects of the mutant Kir6.2, we performed immunohistochemical co-staining experiments with markers of the endoplasmic reticulum (ER) and the Golgi apparatus. Co-staining with antibodies against the calreticulin protein of the ER, revealed a two-fold increase in co-localization of the mutant channel ($42.6\% \pm 8.8\%$, $n = 11$) in comparison to wild type ($22.3\% \pm 7.7\%$, $n = 11$, Fig. 3). The same results were obtained with the co-localization of the Kir6.2 protein with the ER-EGFP (data not shown). In contrast, co-staining with antisera against the Golgi apparatus, showed no difference in comparison to wild type ($1.3\% \pm 0.1\%$, $n=4$ versus $1.7\% \pm 0.3\%$, $n=5$, Fig. 4). We conclude from these experiments that a significant amount of mutant Kir6.2 is retained in the ER and as a consequence there is a reduced expression of the mutant K_{ATP} channel at the cell membrane.

We therefore tested whether the additional mutation of the ER retention signal RKR to AAA rescued K_{ATP} channels. As expected, this led to a significant decrease of fluorescence that co-localized with the ER marker calreticulin ($13.1 \pm 4.9\%$ versus $42.6 \pm 8.8\%$, $n = 8-11$ cells, $p < 0.01$, data not shown). However, even after elimination of the ER retention signal, no K_{ATP} currents were recorded (Fig. 5A-B, $n = 7$, $p < 0.01$). Furthermore diazoxide applied to cells transfected with H259R_{AAA} did still not restore K_{ATP} currents (Fig. 5C). In contrast, in WT_{AAA} transfected cells diazoxide enhanced K_{ATP} currents.

Reduced expression of the mutant K_{ATP} channel at the cellular membrane

This conclusion is further supported by confocal images showing a marked reduction in membrane staining of the mutant Kir6.2 K_{ATP} channel compared to wild type. Co-staining with the plasmic membrane marker, toxin-GPI-alexa546, revealed a reduced co-localization of the mutant channel with the plasma membrane ($9.5\% \pm 1.5\%$, $n = 3$) in comparison to wild type ($14.5\% \pm 1.3\%$, $n = 3$, Fig. 6). Finally, these results were confirmed by total internal reflection fluorescence (TIRF) imaging, which allows to selectively visualize fluorescence localized at the cell surface. Again expressing the mutant KCNJ11 cDNA led to a significantly lower signal of the protein at the cell surface compared to wild type (Fig. 7A). To validate our TIRF experiments and to decrease the likelihood that the fluorescence signal obtained may relate to channels close to the membrane, we transfected HEK293T with two GFP fusion constructs, localizing either to the plasma membrane (Mb-EGFP) or to the ER (ER-EGFP, Ref. 20, Fig. 7B). The fluorescence observed in TIRF with the membrane marker Mb-EGFP is comparable with the fluorescence obtained with Kir6.2 WT. The TIRF fluorescence of the ER-EGFP protein is similar to the one seen with the mutant H259R protein. Taken together, these results thus show that the mutant Kir6.2 is partially retained in the ER, but a fraction still reaches the cell membrane.

Discussion

We describe a human mutation located at the C-terminus of Kir6.2 that impairs trafficking and abolishes channel function. Immunohistochemical visualization of mutated Kir6.2 revealed a decreased surface pool, while fluorescence in the ER was enhanced. In addition whole cell currents were abolished, which suggests that the fraction of channels that

eventually reaches the surface is not functional. This conclusion is also supported by the non-responsiveness to diazoxide in the patch clamp experiments.

During biosynthesis, the Kir6.2 protein is exported from the ER only if properly assembled into an octameric K_{ATP} channel (24). In fact, the formation of the Kir6.2 tetramer leads to the masking of an ER-retention signal located in the cytoplasmic tail of the Kir6.2 protein. Mutation analysis indicate that the ER-retention signals contain a -RKR- motif (24). Assembly of functional channels that reach the surface will only occur after a second retention signal located on the SUR subunit is subsequently masked. An alternate assembly model proposes the formation of a SUR-Kir6.2 heterodimer prior to the octamer formation (17). Our data are compatible with both assembly models. In fact, the H259R mutation may interfere in several ways with this quality control mechanism. First, the H259R mutation may have created a new retention signal, a possibility which however is not very likely since the mutation did not create any of the established retention motifs including -RXR- (26). Second, the -RKR- motif could have been indirectly inactivated by the H259R mutation through a change of the tertiary conformation of the Kir6.2 protein. This would cause an inappropriate trafficking of Kir6.2_{H259R} tetramers to the cell surface without the need to co-assemble with SUR just as C-terminally truncated Kir6.2 proteins are inserted into the membrane (24, 27). Again, this scenario seems unlikely since such C-terminally truncated tetramers constitute functional channels while the H259R mutation led to a complete absence of currents. Therefore, we favor a model whereby the H259R mutation would change the conformation of the Kir6.2 protein in a way that would prevent the complete masking of the retention signal that normally occurs during assembly (24, 28). This model could explain partial retention in the ER that can be overcome by mutating the RKR sequence. As a consequence only a fraction of the mutated channels is expressed at the surface. The mutation may cause structural alteration abolishing function for example by affecting ATP-gating, which may

provide an explanation why even in the presence of surface fluorescence no currents were recorded. In line with the interpretation mutating the ER retention signal RKR in Kir6.2 to AAA did not rescue K_{ATP} currents.

A similar dual defect was reported for the $\Delta F1388$ mutation in the SUR1 subunit; this mutation caused defective trafficking and lack of surface expression (29). Further experiments led to the conclusion that even if expressed at the surface, the $\Delta F1388$ mutation interfered with K_{ATP} function. The severity and the early onset of hypoglycemia in the case described here may reflect the complete loss of channel function revealed in this study. Moreover, the patient was resistant to diazoxide, an observation mirrored by the absence of an effect of diazoxide in the electrophysiological recordings (22).

The observation that diabetes resolved 18 months after subtotal pancreatectomy is unusual and could potentially be explained by a partial regeneration of the pancreas as previously reported (30, 31). We assume that the number of beta cells has increased during pancreas regeneration and that the amount of secreted insulin is sufficient to avoid overt hyperglycemia. The child is still on a strict diet with three main meals and three snacks including one at bedtime, which could help to avoid severe hypoglycemic episodes, secondary to inappropriate insulin secretion. It is possible that our index case experiences unrecognized hypoglycemic episodes.

K_{ATP} channels containing the Kir6.2 subunit are present in the pancreatic beta cell, the brain, the cardiomyocyte and the smooth muscle (32-36); the targeted disruption of Kir6.2 in mice showed exercise induced arrhythmia and sudden cardiac death (1). We therefore recorded an electrocardiogram over a 24-hour period, which included a period of exercise, however no arrhythmia was noted and the QT interval was normal. Despite severe hypoglycemic episodes during infancy, the child is developing normally at four years of age and has a normal statural growth. Loss of function of Kir6.2 in the human brain seems not to

interfere with normal development. It is possible that other inwardly rectifying potassium channels substitute for the neuronal loss of Kir6.2. Kir6.1 however seems an unlikely candidate since it is mainly expressed in astrocytes (37). However the child could be at risk for hypoxia-induced seizures, as described in Kir6.2^{-/-} mice (2). The parents, both heterozygous for the H259R mutation, never experienced hypoglycemic episodes, excluding a dominant negative effect of H259R.

In conclusion, we identify histidine 259 as an important residue in the C-terminus of human Kir6.2 protein that affects trafficking and is required for channel function.

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Figure Legends**Fig. 1**

Identification of a new point mutation H259R in the C-terminus of the Kir6.2 protein in the index patient. Protein sequence alignment of the region containing the mutation shows the conservation of the mutated amino acid between species.

Fig. 2

Functional analysis of mutated K_{ATP} channel. A: Whole-cell currents recorded in response to ramps of voltage from -120 to -40 mV over a 3-s period from voltage-clamped HEK293T cells (holding potential: -80 mV) co-expressing Kir6.2WT + SUR1. B: Kir6.2H259R + SUR1 and Kir6.2H259R + SUR1 after an incubation with 200 μ M of diazoxide for 48 h. Cells were continuously perfused with extracellular solution (see methods) during the course of the recording and 250 μ M of tolbutamide (tolbu) was added when indicated to block recombinant K_{ATP} currents and allow quantification of their amplitude. C: Membrane slope conductance values (G_m as mean \pm SD) calculated for each type of recombinant channel expressed in HEK293T cells (Kir6.2WT + SUR1, $n = 9$ cells; Kir6.2H259R + SUR1, $n = 8$ cells; Kir6.2H259R + SUR1 submitted to a 48 h treatment with diazoxide, $n = 8$ cells; $p < 0.001$).

Fig. 3

Co-expression of WT and mutant K_{ATP} channel with markers for the ER. HEK293T cells were co-transfected with SUR1 and Kir6.2WT or SUR1 and Kir6.2H259R. Double immunofluorescence staining showed a two-fold increase in co-localization of the mutant K_{ATP} channel with the calreticulin protein in the ER (yellow staining, arrow) in comparison to WT. Photomicrographs were imaged confocally.

Fig. 4

Co-expression of WT and mutant K_{ATP} channel with markers for the Golgi apparatus. HEK293T cells were co-transfected with SUR1 and Kir6.2WT or SUR1 and Kir6.2H259R. Double immunofluorescence staining of the mutant K_{ATP} channel with the giantin protein of the Golgi apparatus (yellow staining, arrow) showed no difference in comparison to WT. Photomicrographs were imaged confocally.

Fig. 5

Functional analysis of mutated K_{ATP} channel devoid of the RKR retention signal. A: Whole-cell currents recorded in response to ramps of voltage from -120 to -40 mV over a 3-s period from voltage-clamped HEK293T cells (holding potential: -80 mV) co-expressing either Kir6.2WT_{AAA} + SUR1 or Kir6.2H259R_{AAA} + SUR1. B: Membrane slope conductance values (G_m as mean \pm SD) calculated for each type of recombinant channel expressed in HEK293T cells (Kir6.2WT_{AAA} + SUR1, $n = 8$ cells; Kir6.2H259R_{AAA} + SUR1, $n = 7$ cells; $p < 0.01$). C: Bars implicate application of diazoxide (diazox, 100 μ M) and tolbu (250 μ M). Diazoxide applied to cells transfected with H259R_{AAA} did not restore K_{ATP} currents. In contrast, in WT_{AAA} transfected cells diazoxide enhanced K_{ATP} currents.

Fig. 6

Reduced surface expression of mutant H259R- K_{ATP} channel. HEK293T cells were co-transfected with SUR1 and Kir6.2WT or SUR1 and Kir6.2H259R. Co-localization of the wild type or mutant K_{ATP} channel and the membrane marker, toxin-GPI-alexa546, was visualized by confocal imaging. Double immunofluorescence staining revealed decreased membrane expression (yellow staining, arrow) for the mutant K_{ATP} channel in comparison to WT.

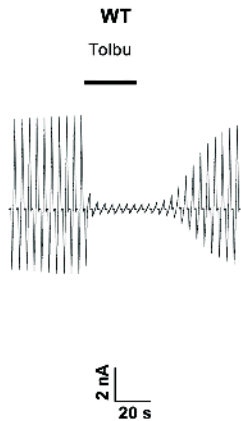
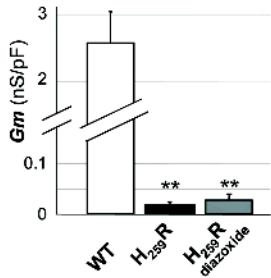
Fig. 7

Wide-field fluorescence and TIRF fluorescence were visualized for the WT K_{ATP} and the mutant K_{ATP} channel. A: The same cell is shown with the wide-field and with TIRF technique (WT in the upper panel and H259R in the lower panel). The TIRF technique showed expression of the WT K_{ATP} channel at the surface (white arrows), whereas the mutant K_{ATP} expression was markedly reduced. B: Wide-field fluorescence and TIRF fluorescence were visualized for the Mb-EGFP and the ER-EGFP. The same cell is shown with the wide-field and with TIRF technique (Mb-EGFP in the upper panel and ER-EGFP in the lower panel). The TIRF technique showed expression of the Mb-EGFP at the surface whereas the ER-EGFP expression at the surface was very weak.

	240	250	260	270	280	290	
Kir6.2_Rabbit	VPLHQVDI	PMENGVGGNS	IFLVAPLII	<u>HH</u> VIDANS	PLYDLA	PSDLHHHQDLEI	IVILEGV
Kir6.2_GuineaPig	VPLHQVDI	PMENGVGGNS	IFLVAPLII	<u>YH</u> VIDANS	PLYDLG	PSDLHHHQDLEI	IVILEGV
Kir6.2_Mouse	VPLHQVDI	PMENGVGGNG	IFLVAPLII	<u>YH</u> VIDSNS	PLYDLA	PSDLHHHQDLEI	IVILEGV
Kir6.2_Rat	VPLHQVDI	PMENGVGGNS	IFLVAPLII	<u>YH</u> VIDSNS	PLYDLA	PSDLHHHQDLEI	IVILEGV
Kir6.2_Human	VPLHQVDI	PMENGVGGNS	IFLVAPLII	<u>YH</u> VIDANS	PLYDLA	PSDLHHHQDLEI	IVILEGV
Consensus	VPLHQVDI	PMENGVGGNS	IFLVAPLII	<u>YH</u> VIDANS	PLYDLA	PSDLHHHQDLEI	IVILEGV

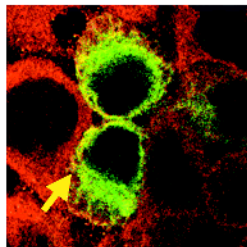
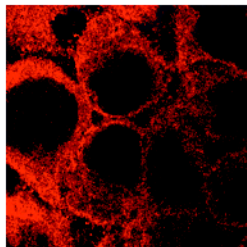
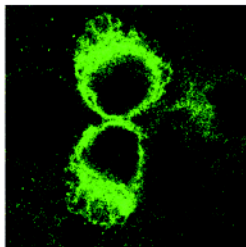


H259R

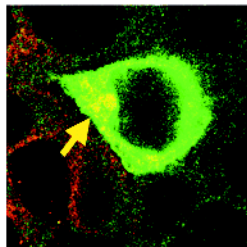
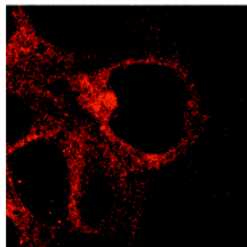
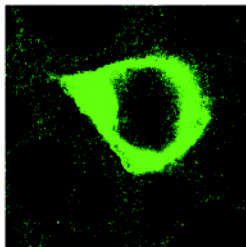
A**B****C**

Sur1 + Kir6.2

WT



H259R



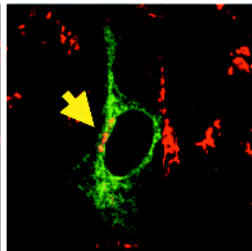
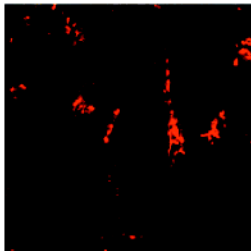
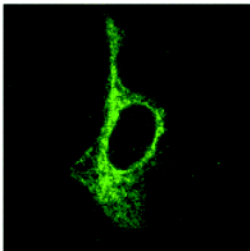
Sur1 + Kir6.2

Kir6.2

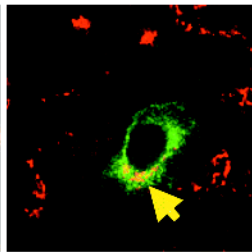
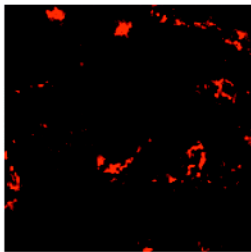
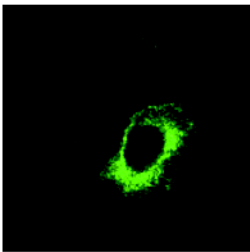
Golgi

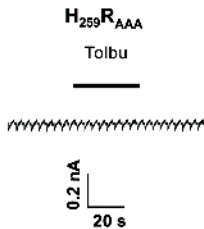
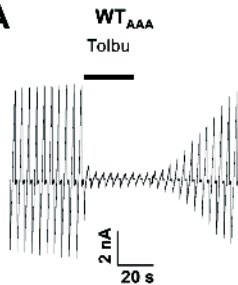
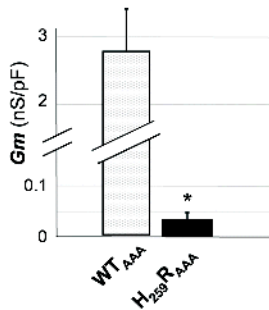
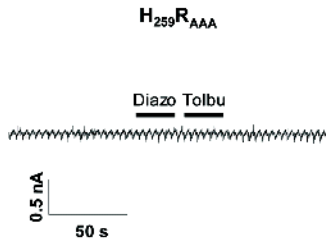
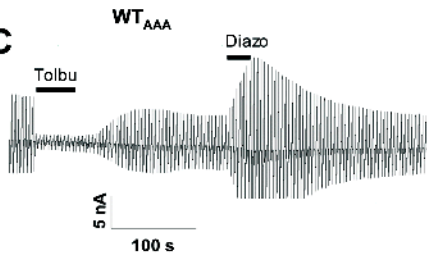
Kir6.2 / Golgi

WT



H259R



A**B****C**

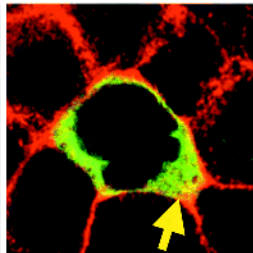
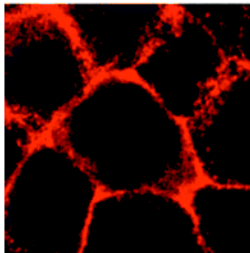
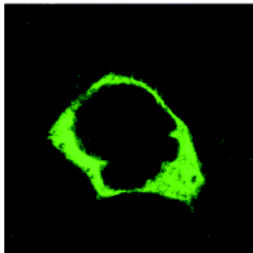
Sur1 + Kir6.2

WT

Kir6.2

GPI

Kir6.2 / GPI



H259R

