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# The novel C-terminal *KCNQ1* mutation M520R alters protein trafficking

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**Abstract**

The long QT-syndrome is characterized by a prolongation of the QT-interval and tachyarrhythmias causing syncopes and sudden death. We identified the missense mutation M520R in the calmodulin binding domain of the Kv7.1 channel from a German family with long QT-syndrome. Heterologous expression of the mutant did not reveal any whole-cell currents independent of the auxiliary subunit KCNE1. Co-expression of the wild-type Kv7.1 channels and the mutant showed that the mutant did not have a dominant negative effect. In immunocytochemical assays of transfected COS-1 cells wild-type Kv7.1 showed an immunopositive labeling of the plasma membrane. For M520R no plasma membrane staining was visible, instead a strong signal in the ER was observed. These results indicate that the LQT1 mutation M520R leads to ER-retention and dysfunctional trafficking of the mutant channel resulting in haploinsufficiency.


*Keywords:* Arrhythmia; Long QT syndrome; KCNQ1; Kv7.1; Trafficking

The congenital long QT-syndrome (LQTS) is a clinically and genetically heterogeneous disease characterized by a prolonged QT-interval and repolarization abnormalities resulting in an increased risk of polymorphic ventricular arrhythmias. These cardiac arrhythmias, typically in the form of *torsade de pointes* may underlie ventricular fibrillation, recurrent syncopes, and sudden death [1]. The disturbance of the ventricular repolarization is caused by mutations in at least nine genes, of which seven encode ion channel subunits. Where *KCNQ1* (LQT1), *KCNH2* (LQT2) and *KCNJ2* (LQT7) encode α-subunits of potassium channels, *SCNA5* (LQT3) codes for a sodium channel and *CACNA1C* (LQT8) codes for a calcium channel. *KCNE1* (LQT5) and *KCNE2* (LQT6) code for accessory subunits which interact with potassium channels. The non-ion channel proteins ankyrin-B (LQT4) and caveolin (LQT9) are required for the localization or regulation of ion channels in cardiomyocytes, respectively [2,3].

*KCNQ1* encodes the voltage-gated Kv7.1 channel which is built by the assembly of four α-subunits through interaction of C-terminal assembly domains (CAD) [4,5]. Co-assembly of the regulatory β-subunit KCNE1 (minK) with the α-subunits results in a significant change of the biophysical properties of the channel complex, resembling the native slowly activating delayed rectifying potassium current $I_{Ks}$ in cardiomyocytes [6,7]. Most of the mutations associated with LQT1 syndrome are located in the membrane-inserted region of the Kv7.1 channel protein. However, several studies showed that also mutations in the N- and C-terminus of the Kv7.1 channel impair Kv7.1 mediated ion currents in various heterologous expression systems. So far, only few mutations in Kv7.1

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displaying trafficking deficiency have been identified compared to a large incidence of mutations in *KCNH2* leading to trafficking defects [8–11].

In the present work we have identified the *KCNQ1* missense mutation M520R from a German family with congenital LQT1 syndrome and mitral valve prolaps. The mutation resides within a region the hKv7.1 C-terminus which has been shown to be a site of calmodulin interaction [12–15]. We investigated the functional properties of this single amino acid exchange in heterologous expression systems. The mutation resulted in non-functional channels, which showed impaired protein trafficking.

## Materials and methods

*Molecular biology.* For DNA diagnosis, informed consent according to the ethics committee of the University of Münster was obtained from all family members. DNA was isolated from venous EDTA blood and direct sequencing of the complete coding sequences of the *LQT1* (*KCNQ1*) and the *LQT2* (*KCNH2*) genes were performed. The mutation M520R of human Kv7.1 (NM_000218) was constructed using site-directed PCR mutagenesis and subsequently cloned into the pcDNA3 and pGEM-HE vectors for expression in mammalian cells or *Xenopus laevis* oocytes, respectively. Human KCNE1 cDNA (NM_000219) was cloned into the pcDNA3 and pGEM-HE vectors. For expression in *Xenopus laevis* oocytes, mRNAs were prepared using the T7 m-Message Machine kit according to the manufacturer's instructions (Ambion). For analysis in the yeast two-hybrid system, sequences corresponding to the N-terminus (aa 1–122) and the C-terminus (aa 354–676) of Kv7.1 wild-type (WT), the C-terminal mutant of Kv7.1 and the C-terminus of Kv7.2 (aa 307–893, Y15065) were amplified by PCR. Fragments were cloned in frame into the GAL4 activation domain (AD) vector, pGAD424 (CLONTECH). Calmodulin (BC011834) and frequenin (NM_024366) were cloned in frame into the GAL4 DNA binding domain (BD) vector, pAS2-1 (CLONTECH). All constructs were verified by sequencing.

*Cell culture.* CHO-K1 and COS-1 cells were cultured in DMEM medium supplemented with 10% fetal calf serum. For the CHO-K1 cells the serum was supplemented with L-proline (20 mg in 500 ml). The cells were grown at 37 °C in a humidified atmosphere of 5% $CO_2$. The cells were transiently transfected with hKv7.1 (WT and/or M520R mutant) with or without hKCNE1. The cells were transfected with 1 μg of plasmid for CHO-K1 and 2 μg for COS-1 using Lipofectamine (Life Technologies), according to the manufacturer's instructions. 48 h post transfection, the cells were trypsinized and transferred to cover slips.

*Electrophysiology.* Whole-cell currents were measured from transiently transfected CHO-K1 cells using an EPC-9 amplifier (HEKA electronics, Germany). Pipettes were pulled from borosilicate glass with a DPM electrode puller (Zeitz Instruments, Germany) and had a resistance of 1.5–2.5 MΩ. The extracellular solution contained (in mM): NaCl 140, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1 and Hepes 10, adjusted to pH = 7.4. The intracellular solution used in the pipettes contained 100 nM free calcium with (in mM) KCl 110, Hepes 10, EGTA 10, $CaCl_2$ 5.17, $MgCl_2$ 1.42. Data were sampled with Pulse (HEKA electronics, Germany) and analyzed with IGOR software (WaveMetrics, Lake Oswego, USA). Data are presented as means ± SEM of $n$ experiments and, where appropriate, have been analyzed using Student's $t$-test.

*Confocal microscopy.* Transiently transfected COS-1 cells were fixed with 4% paraformaldehyde 48–72 h post transfection and labeled with anti-Kv7.1 (Santa Cruz) and anti-phospho-di-esterase (PDI, Biocite) as previously described [16]. All secondary antibodies were from Molecular Probes. Images were obtained using a Leica TCS SP2 confocal laser scanning microscope and treated with the use of MetaMorph imaging software (Universal Imaging) and Adobe PhotoShop 5.5 (Adobe System Inc.).

*Yeast two-hybrid analysis.* cDNA encoding different regions of the Kv7.1 and Kv7.2 channel proteins and CaM were co-transformed into *Saccharomyces cerevisiae* (CG1945) according to the manufacturer's instructions (MATCHMAKER Two-hybrid System, CLONTECH). Co-transformants were tested for protein–protein interaction on medium lacking histidine and incubated for 4–7 days at 30 °C. Negative controls were: (i) transformation of each single fusion protein cDNA construct and each single empty vector; (ii) co-transformation of empty DNA-AD plasmid pGAD424 with CaM or Frq constructs; (iii) co-transformation of empty DNA-BD plasmid pAS2-1 with either KCNQ construct.

## Results

### *Case presentation*

A family of 7 living members was referred for clinical and genetic consultation (Fig. 1); the 34-year-old female proband (III-1) was asymptomatic but had a prolongation of the heart-rate corrected QT-interval (QTc: 460–490 $ms^{1/2}$). At a heart-rate of 54 bpm, the T-wave was small-based and tall, together with an isoelectric ST-segment. Transthoracic echocardiography revealed a discrete mitral valve prolaps. Other examinations including routine physical examination, exercise ECG, EEG, programmed ventricular stimulation, and Holter monitoring, were unremarkable. There was no history of LQTS or sudden death in the family. Investigation of the parent's ECGs revealed a normal ECG in the father (II-1; QTc: 372 $ms^{1/2}$), but a QTc prolongation in the ECG of the 50-year-old, asymptomatic mother (II-2; 54 bpm, 466 $ms^{1/2}$) together with an isoelectric ST-segment. The mother received no (anti-adrenergic) therapy and also had a mitral valve prolaps. The aunt (II-2, 56 years, no medication) had a similar clinical presentation, being asymptomatic so far, a mitral valve prolaps and a borderline QTc value (460 $ms^{1/2}$). The elder cousin (III-3) was also genetically tested, but clinical information was not provided. The asymptomatic younger cousin (III-3) had a similar repolarization phenotype (464 $ms^{1/2}$).

### *Electrophysiological characterization of hKv7.1-M520R*

To define the molecular mechanism of the LQT1 missense mutation we transiently expressed wild-type (WT) and mutant Kv7.1 in CHO-K1 cells. Expression of the Kv7.1-WT channel gave rise to relatively slowly activating and deactivating potassium currents (Figure S1A, at +100 mV: 982 ± 256 pA, $n = 16$). In contrast, expression of Kv7.1-M520R channels induced currents very similar to untransfected CHO-K1 cells (Figure S1C, at +100 mV: 184 ± 28 pA, $n = 14$ vs. 104 ± 8 pA, $n = 16$, respectively). In order to mimic the heterozygous state in the patient we co-transfected Kv7.1-WT and Kv7.1-M520R in a 1:1 ratio (Figure S1B). The current recorded was about half of the one recorded in CHO-K1 expressing Kv7.1-WT only (614 ± 221 pA, $n = 12$). Thus, the residual current was mostly due to expression of Kv7.1-WT.

In cardiomyocytes, Kv7.1 is co-expressed with KCNE1 and together they give rise to the $I_{Ks}$ current. Therefore,

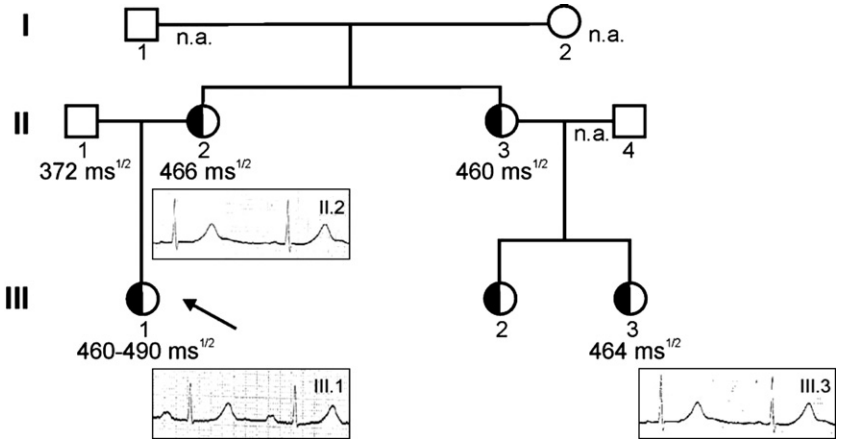


Fig. 1. Clinical characterization. Pedigree structure of a three generation family affected by LQT1-syndrome with given QTc values and their associated ECGs (lead II). Family members harboring the heterozygous mutation M250R are indicated by a half-filled circle.

we co-expressed both channel subunits. As expected, co-expression of Kv7.1-WT and KCNE1 elicited the typical $I_{Ks}$ current with a sixfold increase in current (Fig. 2A). Co-expression of Kv7.1-M520R with KCNE1 did not induce any $I_{Ks}$-like currents ($166 \pm 26$ pA, $n = 10$, Fig. 2C) while co-transfection of Kv7.1-WT, Kv7.1-M520R and KCNE1 gave rive to $I_{Ks}$-like currents with about half amplitude compared to Kv7.1-WT and KCNE1 co-expression ($3479 \pm 869$ pA, $n = 21$ vs. $6058 \pm 2241$ pA, $n = 19$, respectively, Fig. 2B and D). Hence, the mutant produced neither functional channels when expressed alone nor did it display a dominant-negative phenotype when co-expressed with wild-type channels. As it has been shown that $I_{Ks}$ current properties may vary with the expression system used [17], and lower temperatures may rescue trafficking defects [18], we validated our results with expression in *Xenopus laevis* oocytes, however, we did see the same (data not shown).

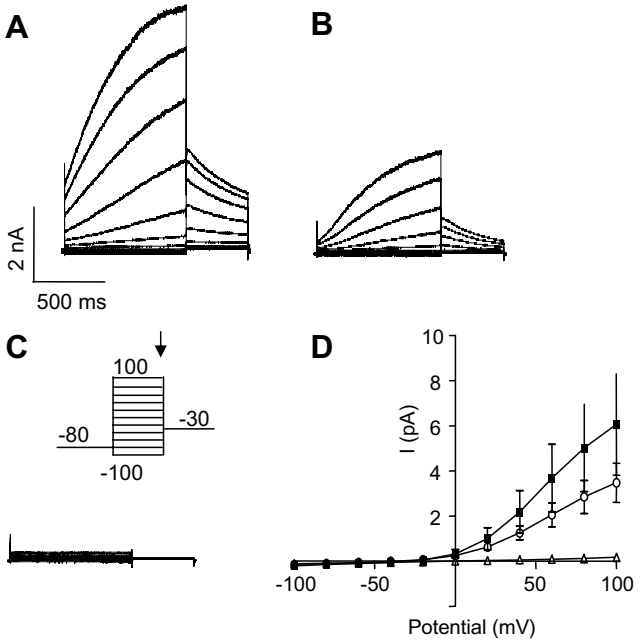


Fig. 2. Electrophysiological characterization of Kv7.1-WT and Kv7.1-M520R in the presence of KCNE1. CHO-K1 cells were transiently transfected and whole-cell currents were measured using single-electrode voltage-clamp. Currents were elicited using the voltage-clamp protocol shown in the insert in (C). (A) Kv7.1-WT and KCNE1. (B) Kv7.1-WT + Kv7.1-M520R in a 1:1 ratio and KCNE1 and (C) Kv7.1-M520R and KCNE1. (D) The current–voltage relationships measured at steady-state. Filled squares: Kv7.1-WT and KCNE1, $n = 19$. Open circles: Kv7.1-WT + Kv7.1-M520R and KCNE1, $n = 21$ Open triangles: Kv7.1-M520R and KCNE1, $n = 10$. Mean values ± SEM are shown.

Since the mutation is located in a region of the channel that has been shown to associate with calmodulin (CaM) (see below), we investigated if co-expression with CaM could rescue the electrophysiological phenotype or the trafficking of the channel as had been shown for defective CaM-binding mutants [13]. However, we did not see any increase in current upon co-expression of the calcium sensor (data not shown).

### *Cellular localization*

We investigated if the mutation impedes the subcellular localization of the channels using immunocytochemical analysis of transfected COS-1 cells. Fig. 3 (upper panel, left) shows a strong labeling of the plasma membrane with some cytoplasmic staining due to overexpression for the WT channel. This suggests that WT channels are targeted efficiently to the plasma membrane as shown previously [19]. In contrast, the subcellular localization of the M520R mutant was seriously perturbed (Fig. 3, lower panel, left). No plasma membrane staining was visible, but a strong signal within the cytoplasm was observed. In order to refine the localization within the cytoplasm, we performed co-stainings with the ER-marker protein-disulphide-isomerase (PDI) (Fig. 3, middle). The overlay picture shows a clear co-localization of the mutant channel with the ER-marker (Fig. 3, right). These results strongly indicate that the LQTS mutation M520R leads to ER-retention and dysfunctional trafficking of mutant channel subunits.

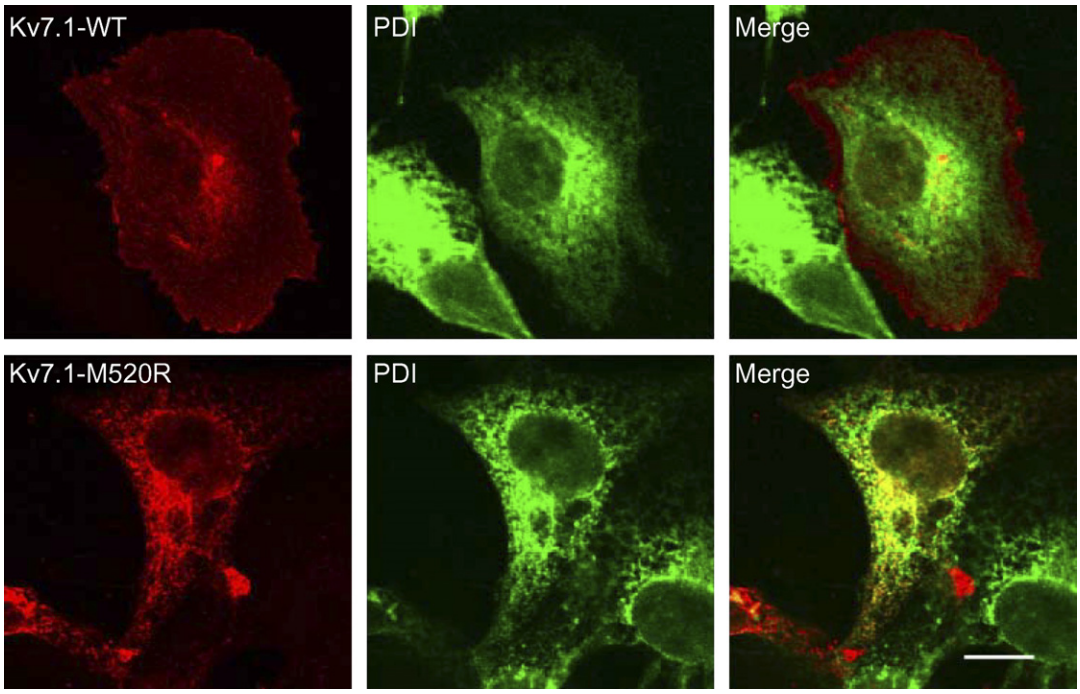


Fig. 3. Subcellular localization of Kv7.1-WT and Kv7.1-M520R. COS-1 cells were transiently transfected with Kv7.1-WT (upper panel) or Kv7.1-M520R (lower panel). The cells were co-stained with a Kv7.1 antibody (left column) and with an antibody directed against the ER marker PDI (central column). Overlays of the two images are shown in the right column. The scale bar is 10 μm.

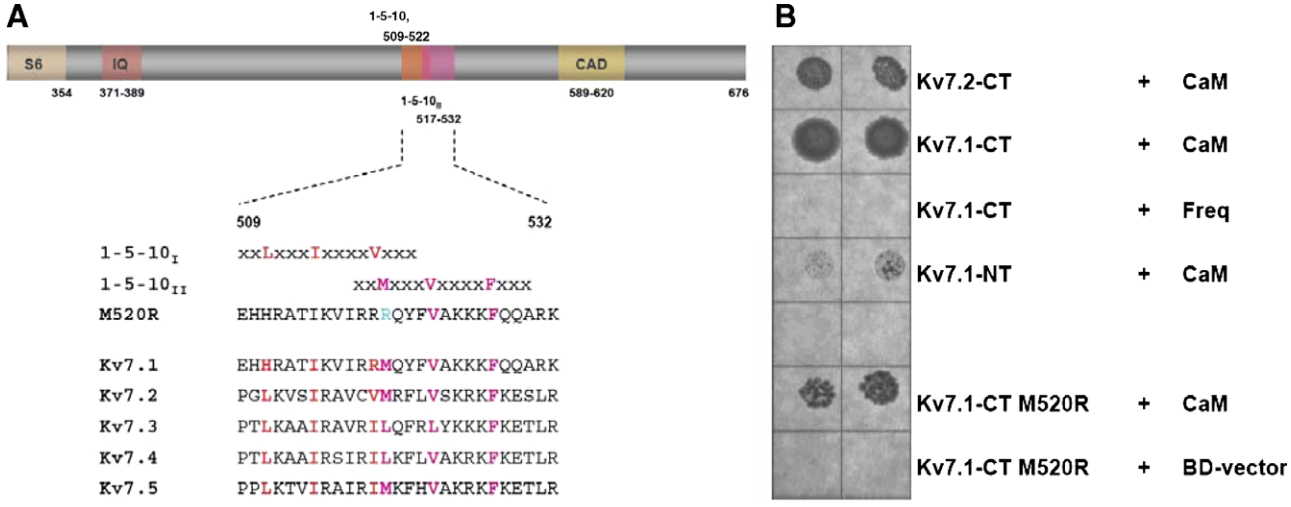


Fig. 4. Calmodulin binding to the C-terminus of the Kv7.1 channel. (A) Domain structure (IQ, IQ-calmodulin binding motif; CAD, C-terminal assembly domain, see text for references) and alignment of the proposed CaM interacting helix B and position of the M520R mutant. (B) Detection of CaM binding to WT or mutant Kv7.1 using the yeast two-hybrid system: CaM binding was assayed by *HIS3* induction in the GAL4 based yeast two-hybrid system (strain *S. cerevisiae* CG1945). Double determination of growth on selective medium is shown (CaM, Calmodulin; Freq, Frequenin; CT, C-terminus; NT, N-terminus).

### *Calmodulin binding assay*

The mutation M520R lies within a region of the Kv7.1 channel which has been shown to be involved in the interaction with the calcium binding protein calmodulin with Kv7 channels [12–15]. Using secondary structure prediction algorithms and several biochemical assays it was shown that an IQ-motif for CaM binding in helix A and two overlapping consensus 1–5–10 CaM binding motifs in helix B were necessary for interaction of different Kv7 channels with the calcium sensor (Fig. 4A) [12–15,20].

To test if the mutant M520R disrupts channel function by impairing the interaction of Kv7.1 with CaM, we performed yeast two-hybrid experiments (Fig. 4B). The C-termini of Kv7.1-WT and Kv7.1-M520R were co-transformed with CaM and analyzed for activation of the *HIS*

reporter gene in yeast. The interaction of the Kv7.2 C-terminus with CaM was used as positive control. As negative controls co-transformations of either the Kv7.1 N-terminus with CaM or of the C-terminus with frequenin (Frq), another member of the calcium binding protein family, were performed [21]. WT and mutant Kv7.1 C-termini and Kv7.2 C-terminus did interact with CaM as assayed by *HIS3* induction in the GAL4 based yeast two-hybrid system (Fig. 4B) while the negative controls did not produce any signal. Compared to the WT C-terminus, the mutant showed a slight attenuation of growth possibly indicating sensitivity of this mutant in the Kv7.1 C-terminus for binding of CaM.

## Discussion

We identified the novel *KCNQ1* M520R mutation that co-segregates in a patient diagnosed with LQT1-syndrome. Analysis of family members showed that the heterozygous mutation carriers are characterized by a mild QTc prolongation, an isoelectric ST-segment together with a heart-rate in the lower normal range and a mitral valve prolaps indicating an intermediate probability for familial LQT-syndrome. Our heterologous ion channel expression data show that homomeric mutant channels failed to conduct any current in CHO cells. Co-expressing WT and mutated subunits led to an approximately 50% reduction of Kv7.1 current independent of KCNE1 co-expression, indicating that the mutated subunits do not affect the Kv7.1-WT subunits and resulting in haploinsufficiency on the patient's level. The mutation appears clinically benign, since even female mutation carriers were asymptomatic in the elderly. It has been suggested that the contribution of the $I_{Ks}$ current to the repolarization of the normal ventricular action potential (AP) may be rather limited in the absence of sympathetic stimulation. However, when the AP duration is prolonged e.g. by a reduction in $I_{Kr}$ or during increased sympathetic stimulation, the $I_{Ks}$ current limits action potential duration [22,23]. A reduction of the $I_{Ks}$ by the M520R mutation could therefore only cause an impairment of ventricular repolarization under these conditions, explaining the mild phenotype of the patient.

Immunofluorescence experiments did not show any staining of the mutant Kv7.1 in the plasma membrane in accordance with the absence of $I_{Ks}$ current in the electrophysiological measurements. Co-staining with an ER marker indicated that M520R was trapped in the ER, i.e. the channel trafficking was affected. This mechanism seemed to be different from ER retention/retrieval signals as none of consensus sites for ER retention were present in this region [24]. So far, only a few LQT1 mutants were reported to be trafficking defective. All of them lead to a complete loss of function of the mutant protein due to defective membrane insertion without affecting the remaining WT channel subunits [9,11,25–27]. Recently, Aizawa et al. [28] reported a truncation mutation in the hydrophobic core to cause a dominant-negative suppression of Kv7.1-WT currents due to a trafficking effect. However, those mutant proteins might cause nonspecific disruption of transport that leads to aberrant trafficking of other proteins destined for the plasma membrane or secretion [29].

ER retention may also results from misfolding due to impaired interaction with signaling or scaffolding proteins. The mutation is located within the C-terminus of the Kv7.1 protein which is highly conserved within the Kv7 channel family and harbors several domains responsible for subunit assembly [4,30], processing [9] or interaction with other signaling molecules like yotiao, an AKAP protein [31]. Furthermore, two CaM binding motifs have been examined in the Kv7 C-termini, the IQ-motif and the overlapping 1–5–10 motifs [12–15,20,32], with the mutation M520R residing in the latter. It has previously been suggested that the role of CaM may be in mediating assembly or expression of Kv7 [14,15] similar to what has been shown for the small and intermediate conductance potassium channels, SK and IK [33,34]. Therefore, we considered the M520R mutation to affect the CaM-Kv7.1 interaction. As suggested by secondary structure prediction, the mutation residue is likely to disrupt the amphipatic a-helical structure in helix B, which appears to be necessary for proper interaction of CaM with Kv7.2 [12–15,20,32]. In our yeast two-hybrid assay, we could detect a slight attenuation of the *HIS3* reporter gene activation which might point to an impaired interaction of the calcium sensor with the mutated C-terminus. However, the mutant channel seemed still be able to interact with CaM, possibly indicating that the integrity of the IQ-motif is sufficient to provide interaction with CaM. As it has been shown that there is an intense competition for CaM [35,36], we co-expressed CaM in order to provide sufficient CaM to bind to the channels, however, neither the electrophysiological phenotype nor the expression level of the mutant channel could not be rescued (data not shown). Thus, the functional data suggest that the pathogenesis of the mutation in this study differs from mutations in the same area described in previous reports, in which the mutations with abolished interaction with CaM additionally conferred dominant-negative suppression on the current when co-expressed with WT channel subunits [12] or displayed dramatic changes in inactivation [13,15].

Thus, although the mutation investigated in this study is located in a well characterized region of the channel, the genotype–phenotype correlation does not appear to be simple and further studies are necessary to understand the precise molecular mechanism underlying clinical phenotype variations.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.04.127.

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