Functional evaluation of human ClC-2 chloride channel mutations associated with idiopathic generalized epilepsies

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Running title: ClC-2 mutations in idiopathic generalized epilepsy

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Summary.

The ClC-2 Cl⁻ channel has been postulated to play a role in the inhibitory GABA response in neurons or to participate in astrocyte-dependent extracellular electrolyte homeostasis. Three different mutations in the CLCN2 gene, encoding the voltage-dependent homodimeric ClC-2 channel, have been associated with idiopathic generalized epilepsy (IGE). We study their function in vitro by patch-clamp and confocal microscopy in transiently transfected HEK-293 cells. A first mutation predicts a premature stop codon (M200fsX231). An altered splicing, due to an 11 bp deletion in intron 2 (IVS2-14del11), predicts exon 3 skipping (Δ74-117). A third is a missense mutation (G715E). M200fsX231 and Δ74-117 are non-functional and do not affect the function of the normal (WT) channel. Neither M200fsX231 nor Δ74-117 reach the plasma membrane. Concerning the IVS2-14del11 mutation, we find no difference in the proportion of exon-skipped to normally spliced mRNA using a minigene approach and, on this basis, predict no alteration in channel expression in affected individuals. G715E has voltage- and intracellular Cl⁻-dependence indistinguishable from WT channels. ClC-2 channels are shown to be sensitive to intracellular replacement of ATP by AMP, which accelerates the opening and closing kinetics. This effect is diminished in the G715E mutant and not significant in WT+G715E co-expression. We do not know if in a situation of cellular ATP depletion, this might become pathological in individuals carrying the mutation. We postulate that loss of function mutation M200fsX231 of ClC-2 might contribute to the IGE phenotype through a haploinsufficiency mechanism.

Keywords: Epilepsy; CLCN2 gene; ClC-2 chloride channel; AMP and ATP regulation
Human epilepsy is a heterogeneous disorder defined by recurrent unprovoked seizures affecting about 1-3% of the population during their lifetimes (19). The clinical manifestation of the disease is the consequence of abnormal, synchronized neuronal discharges in the brain, reflecting an imbalance between excitatory and inhibitory influences in a neuronal network. Idiopathic epilepsy lacks clinical and laboratory evidence of central nervous system disease or injury, accounts for approximately 40% of the cases and is of presumed genetic origin. Most idiopathic epilepsies in humans have complex inheritance patterns, except for a few that show Mendelian inheritance and are associated with single gene mutations. Almost all such mutations have been found in genes encoding ion channel proteins -both voltage-gated and ligand-gated- resulting in hyperexcitability. These channel-related epilepsies have been reviewed (29; 35). Mutations of Na+ \((SCN1A, SCN1B \text{ and } SCN2A)\) and K+ channels \((KCNA1, KCNQ2 \text{ and } KCNQ3)\) are associated with certain forms of generalized epilepsy and infantile seizure syndromes. Ligand-gated ion channels, such as nicotinic acetylcholine \((CHRNA4 \text{ and } CHRNB2)\) and GABA receptor subunits \((GABRA1 \text{ and } GABRG2)\), are associated with specific syndromes of frontal and generalized epilepsies, respectively.

ClC-2 belongs to a family of chloride channels that is widely expressed, showing a relatively high level in brain and epithelia (22; 43). In brain, the ClC-2 transcript and protein are present in neurons and astrocytes (16; 38; 40). Using immune electron microscopy, the protein was found in hippocampus, in the soma and dendrites of CA1 and CA3 pyramidal cells and in some interneuronal cells, but was absent from dentate granular cells. ClC-2 immunostaining was seen close to presumed GABAergic inhibitory
synapses. In astrocytes ClC-2 protein has been found at the end feet of astrocytes contacting blood vessels and neurons close to inhibitory synapses (38).

Inwardly rectifying ClC-2-like currents activated by hyperpolarization have been recorded in hippocampal pyramidal cells (40; 41) and in astrocytes (14; 27; 28; 33). In these two cell types the channel might play different roles. In neurons, it is speculated that because of its activation by intracellular Cl\textsuperscript{−}, ClC-2 would prevent accumulation of this anion above equilibrium (41; 42). Intracellular accumulation of Cl\textsuperscript{−} might take place during high frequency stimulation due to an increase in extracellular K\textsuperscript{+} with reversal of KCl cotransport (7; 23). Under these conditions, GABA\textsubscript{A} receptor activity would become excitatory. It must be pointed out that a mechanism involving ClC-2 in preventing a depolarizing GABA response requires Cl\textsuperscript{−} accumulation above equilibrium as a channel is, obviously, not able to extrude ions. According to this reasoning, loss of function mutations of the ClC-2 channel could result in increased excitability in certain neurons.

Hyperpolarization-activated chloride currents are also present in cultured or freshly isolated cortical astrocytes incubated with dibutyryl-cyclic-AMP, co-cultured with neurons or in acute brain slices. These currents were sensitive to physiological changes in extra- and intracellular pH (15; 27), were activated by hyposmotic swelling (13) and were absent in tissues from ClC-2-null mice (28). These currents could have roles in K\textsuperscript{+} buffering, pH regulation and volume regulation (45). Changes in any of those functions could alter the neuron microenvironment and promote changes in excitability.
Recently, a genome wide search study identified a susceptibility locus for idiopathic generalized epilepsy (IGE) on chromosome 3q26 (34). This is the location of the \textit{CLCN2} gene, encoding for the voltage-dependent ClC-2 chloride channel, thus constituting a plausible epilepsy candidate gene. In fact, three different mutations on this channel were found that co-segregated with heterogeneous types of IGE with an autosomal dominant inheritance (18). Two of the mutations predict a truncated protein and the skipping of exon 3 respectively, and they were shown to exert dominant negative effects leading to complete loss of channel function. A third, missense mutation, produces an amino acid replacement (G715E) in the C-terminus, which is associated with a gain of function, allowing the channel to be conductive at reduced intracellular Cl\(^{-}\) concentration. Loss of function would account for hyperexcitability for the first two mutations. A different effect was hypothesized for the third mutation: intense GABAergic and glutamatergic activation could increase intracellular Cl\(^{-}\) through GABA\(_A\)-mediated influx in a glutamate-depolarized situation. During repolarization Cl\(^{-}\) might transiently be found above equilibrium. The presence of active G715E, but not of WT, channels at low intracellular Cl\(^{-}\), would result in Cl\(^{-}\) efflux, depolarization and hyperexcitability of the post-synaptic membrane. This gain of function was proposed to explain hyperexcitability leading to seizures in heterozygous affected patients (18).

All eukaryotic ClC proteins have a long carboxy-terminal cytoplasmic region that contains two CBS (cystathionine beta synthase) domains. These domains, typically 60 residues in length, are present in tandem pairs in a diversity of proteins from archaeabacteria to eukaryotes (4). Several functions have been proposed for CBS domains,
which range from roles in the oligomerization and allosteric regulation of cystathionine-\(\beta\)-synthase, to the subcellular localization, trafficking and gating of CIC channels. The biological importance of these domains, on the other hand, is revealed by point mutations in CBS domains of several unrelated proteins that result in various human inheritable diseases (see (12) and references cited therein). Recently Scott et al. (37) have found that pairs of CBS sequences derived from different proteins bind derivatives of adenosine and importantly, mutations in these domains that cause different hereditary diseases impair this binding. A GST fusion of the isolated CBS domain pair from CIC-2 binds ATP with a \(K_D\) of 1 mM, whilst introduction of the epileptogenic G715E mutation (18) shifted \(K_D\) to 10.4 mM. The authors suggest a functional role of these CBS domains in sensing the energy status of the cell similar to the enzyme AMP-activated protein kinase (AMPK).

In the present study we partially challenge some of the conclusions of Haug et. al. concerning the functional effects of the mutations described, by using patch clamp and confocal microscopy. Additionally, we test the effects of ATP and AMP on CIC-2 electrophysiological properties. We found that intracellular AMP affects the channel kinetic increasing its rate of activation and deactivation. The G715E missense mutation, located between both CBS domains, significantly decreased the effect of AMP compared to WT channels.
Methods

**Plasmid construction.** The mutations found by Haug et al. (18) were generated on a human cDNA ClC-2 clone derived from Caco-2 cells by PCR using the overlap extension method (21) and verified by sequencing. The mutations generated were: a single-nucleotide insertion 597insG resulting in a premature stop codon (M200fsX231), a point mutation G2144A resulting in the substitution of glutamate for glycine (G715E) and the splice variant lacking exon 3 (derived from IVS2-14del11 mutation), corresponding a deletion of the amino acids 74 through 117 (Δ74-117). All the mutated cDNAs were subcloned in the pCR3.1 vector (Invitrogen). After removing the stop codon and introducing an appropriate restriction site, hClC-2 (WT), M200sfX231 and the Δ74-117 mutants were subcloned in frame in the pEGFP-N1 vector (Clontech). We have adopted the nomenclature used by Haug et al. (18) to describe the mutants used in the study and we add the characters -GFP to denote the fusion proteins with the green fluorescent protein.

**Minigene construction and expression.** A 963 bp fragment of the human genomic CLCN2 sequence, containing exons 2, 3 and 4 and the corresponding introns was cloned into the pCR3.1 vector. The 963 bp genomic sequence was generated from genomic DNA isolated from Caco-2 cells using PCR and specific primers located in exon 2 (sense: 5’-AGATGTATGGCCCGGTACACTCAGG-3’) and exon 4 (antisense: 5’-TCAGGCTGTCCGGTATGTTAGAA-3’). Subsequently, the 11 bp deletion in intron 2 found by Haug et al. (IVS2-14del11 mutation) was performed by PCR and both
constructs were sequenced. The expression of both minigenes, WT and intron2Δ11bp, was studied by RT-PCR in the following cells lines: N2a neuroblastoma cells, GT1-7 mouse hypothalamic cells (30) and HEK-293 cells. The cells were transfected with each minigene (1μg of plasmid DNA) using Lipofectamine (Invitrogen) and 48 hrs later total RNA was extracted using Trizol (Invitrogen) and reverse transcription was performed using Superscript II (Invitrogen). Thirty PCR cycles (15 s at 94°C, 30 s at 58°C, 60 s at 72°C) were performed using cDNA of the transfected cell lines with the sense primer located at the start of exon 2 (5'-ATGTATGGCCGGTACACTCAGG-3'), and the antisense primer in the pCR 3.1 vector (5'-TAGAAGGCACAGTCGAGG-3'). PCR reactions were in the linearly increasing phase of amplification under these conditions, as verified by using different number of PCR cycles (20-40 cycles). PCR products were viewed on a 1.5% agarose gel by ethidium bromide staining and the amplicon intensity was quantified using Scion Image software.

Confocal microscopy localization experiments. HEK 293 cell were transiently transfected with 1 μg of the corresponding hClC-2 WT-GFP, M200sfX231-GFP or Δ74-117-GFP plasmid on glass cover slips and 24 h after transfection were mounted in a perfusion chamber for observation. The plasma membrane was stained by incubation with 10μM 1-(3-sulfonatopropyl)-4-[[β[2-(di-n-octylamino)-6-naphthyl] vinyl] pyridinium betaine (di-8-ANNEPS, Molecular Probes) during 2 min. The location of fusion protein was analyzed with a Zeiss LSM 510 confocal microscope equipped with Ar (488 nm) and HeNe (543 nm) lasers. The objective lens used was c-apocromatic 63x/1.2W corr. The excitation of GFP and di-8-ANEPPS was done simultaneously by the 488 nm laser beam
by single-track function. The beam path was set using a 488 nm main dichroic mirror and a 545 nm secondary dichroic mirror. The light emitted for GFP was detected in channel 2 with a 505-530 nm band pass filter and di-8-ANEPPS in channel 1 with a 560-615 nm band pass filter. The image size was 1024 x 1024 pixels and the pinhole setting was 110 (corresponding to 0.87 and 1.0 Airy units for channels 1 and 2, respectively). The images were processed using Huygens Professional deconvolution software.

*Electrophysiological methods.* HEK-293 cells were grown and transiently transfected with expression plasmids for the hClC-2 constructs and πH3-CD8 (1 μg/0.3μg) to identify effectively transfected cells as described previously (6). In co-expression experiments the plasmid constructs were transfected in 1:1 ratio (0.5 μg each). Experiments were performed on cells in 35-mm cell-culture plastic Petri dishes mounted directly on the microscope stage. The bath solution contained (in mM) 140 NaCl, 2 CaCl₂, 1 MgCl₂, 22 sucrose and 10 HEPES pH 7.4 adjusted with Tris. The pipette solution (35 mM Cl⁻) contained (in mM) 100 Na gluconate, 33 CsCl, 1 MgCl₂, 2 EGTA, 1 Na₃ATP and 10 HEPES pH 7.4 adjusted with Tris. Low Cl⁻ solution (10 mM) was prepared by replacement of CsCl with Na gluconate. In some experiments, ATP was replaced with 2 mM AMP. In experiments lacking nucleotides the MgCl₂ concentration was 0.25 mM or 1 mM, the calculated free Mg²⁺ in the pipettes solutions containing 1 mM ATP and 2 mM AMP respectively. Liquid junction potentials were calculated (3) and appropriate corrections applied. Standard whole cell patch-clamp recordings were performed as described elsewhere (9) using an Axopatch 200B (Axon Instruments, USA) or an EPC-7 (List, Germany) amplifier. The bath was grounded via an agar-150 mM KCl
bridge. Patch-clamp pipettes had resistances of 2-3 MΩ. The voltage pulse generator and analysis programs were from Axon Instruments. When giving trains of pulses, an interval of 60 s or 90 s between pulses was left at the holding potential to allow for complete current deactivation. In some experiments, a ramp taking the voltage from -130 to 30 mV in 50 ms was given after each pulse to test for selectivity conservation. Experiments where a change in $E_{rev}$ was seen were discarded. The currents generated by transfection were neither observed in untransfected cells nor in cells transfected with the $\pi$H3CD-8 plasmid alone. There was no detectable functional difference between constructs encoding WT or mutant ClC-2 channels and their respective fusion proteins with GFP.

Time courses for current activation and deactivation were described by fitting a double exponential plus a constant term equation (6) of the form:

$$I(t)/I = A_f \exp(-t/\tau_f) + A_s \exp(-t/\tau_s) + A_0$$

(1)

Where $I(t)$ is current as a function of time and $I$ is current at steady-state, for activation, or at time zero, for deactivation. To obtain an estimate of apparent open probability, tail currents as function of voltage were adjusted by a Boltzmann distribution of the form:

$$G = G_0 + (G_{\text{max}} - G_0) / \{1 + \exp[(V - V_{0.5})/k]\}$$

(2)

Where $G$, $G_0$, and $G_{\text{max}}$ are conductance as a function of voltage, residual conductance independent of voltage, and maximal conductance at full activation (extrapolated),
respectively. $V_{0.5}$ is the voltage at which 50% activation occurs, and $k$ is the slope factor. The traces used for the analysis were taken at least 10 min after establishing the whole-cell recording mode.
Results

The M200fsX231 mutant of hClC-2 predicts a truncated protein lacking 13 out of 18 expected membrane helices including most putative pore-forming regions. Not surprisingly, and as reported before (18), expression of a M200fsX231 construct in HEK-293 cells did not yield any significant Cl- currents (n=13, not shown). Co-expression of WT-GFP and M200fsX231 cDNAs in a 1:1 ratio, on the other hand, produced sizable currents with voltage-dependence similar to that found when expressing WT-GFP on its own (Fig. 1A). Absolute values of conductance obtained are shown in Fig. 1B. On average, a lower maximal conductance ($G_{\text{max}}$) was obtained for the WT-GFP +M200fsX231 co-expression, a difference that did not reach statistical significance in the dispersion of the data.

A second mutation consists of an 11 bp deletion (IVS2-14del11) in intron 2 close to the splice acceptor site. Haug et al. (18) suggested that this mutation would lead preferentially to an alternatively spliced mRNA. The putative protein variant, Δ74-117, would lack most of α-helix B, the largest α-helix predicted to lie at the interface between the channel and the membrane (10). We confirm (n=23, not show) previous data showing that such a construct has no channel activity (18). Co-expression of the mutant cDNA together with the WT channel did not alter the characteristics of the last. This is shown in Fig. 1C that demonstrates that the voltage-dependence of currents encountered after WT and WT+Δ74-117 transfections did not differ significantly. Fig 1D shows that high $G_{\text{max}}$
was attained in both types of experiment, suggesting that co-transfection with Δ74-117 did not affect the expression of the WT current.

It is intriguing that the Δ74-117 mRNA has been found to be present in peripheral blood cells of both patients and healthy controls, albeit at a lower level in the latter (18). We reasoned that perhaps a cell-specific splicing might favor the Δ74-117 mRNA in cells of neural origin. We constructed two minigenes as illustrated in the scheme in Fig. 1E, one containing a normal intron 2 and one carrying the 11 bp deletion. These were transfected into N2a neuroblastoma cells, GT1-7 mouse hypothalamic cells and HEK-293 cells, and their processing was assessed by amplifying the RNA products by RT-PCR. The results are shown in the gel for N2a cells. Both minigenes generated the RNAs of the expected three-exon size (495 bp), suggesting that the splicing machinery recognized the genomic structure of these constructs. A product of the expected, exon-3-skipped size was also observed (365 bp). Sequencing of the amplicons confirmed their identities with RNAs stretches corresponding to Exon2/3/4 and Exon2/4. Using a semi-quantitative RT-PCR approach we demonstrated that 495 and 365 bp products were generated in similar ratios independently of which minigene was used in the experiment. In N2a cells the fraction of exon 3 skipping was 0.38 ± 0.03 and 0.31 ± 0.03 (mean ± SEM, n=7) for minigenes WT and intron2Δ11bp respectively. This difference did not reach statistical significance by t-test (p=0.1). Experiments with GT1-7 and HEK-293 cells gave similar results (data not shown).
It has been claimed that both M200fsX231 and Δ74-117 mutants of ClC-2 reach the membrane to exert dominant negative effects that inhibit the activity of WT ClC-2 markedly (18). In view of our results, we re-examined the issue of the localization of these mutants and compared it to that of WT ClC-2. Green Fluorescent Protein (GFP) fusion protein distribution was compared to that of the membrane dye di-8-ANEPPS. Fig. 2A-C shows WT ClC-2-GFP, di-8-ANEPPS and merged images respectively. ClC-2-GFP (A, green) had a wide intracellular distribution and what appeared as discrete peripheral labeling that might correspond to the plasma membrane. Comparison with di-8-ANEPPS distribution (B, red) and, particularly, examination of the merged image (C) revealed discrete areas of superposition (yellow). The distribution of M200fsX231-GFP and Δ74-117-GFP fusion proteins is shown in Figs. 2D-F and 2G-I respectively. The fluorescence from M200fsX231-GFP and Δ74-117-GFP was observed throughout the cell interior and no superposition with the membrane dye di-8-ANEPPS was detected.

In addition to their dependence on hyperpolarization, ClC-2 channels are gated by intracellular Cl⁻. Mutation G715E has been reported not to affect ClC-2 current amplitude but to have altered Cl⁻-dependent gating (18). We have re-examined this issue and find, as reported, that G715E mutant produced sizable currents with apparently normal gating behavior. We studied voltage-dependent gating at two intracellular Cl⁻ concentrations. Fig. 3A and B show that decreasing Cl⁻ from 35 to 10 mM produced the expected shift in $V_{0.5}$, but there was no significant difference between WT and G715E mutant channels. The $V_{0.5}$ values at 10 mM intracellular Cl⁻ are less well defined that those at higher concentrations as complete activation was not reached. In experiments at 10 mM Cl⁻, tail
currents elicited after a -90 mV pulse were 499 ± 76 (n=5) and 352 ± 115 (n=6) pA for WT and G715E respectively. This indicates that at physiological membrane potential there was no effect of the mutation on ClC-2 activity. The kinetics of activation of WT and mutated channel was also examined by fitting equation (1) to the current relaxations. Time constants for activation are shown in Fig. 3C with the weight of different components to the fit in Fig. 3D-F. There was no significant difference in any of the parameters between WT and G715E channel.

ClC-2 has two so-called CBS domains in tandem in its C-terminus. These conserved sequences are believed to form AMP or ATP binding sites and act as regulators of protein function. Mutation G715E has been proposed to affect the binding of nucleotides by the CBS domains in ClC-2 (37). The experiments shown in Fig. 3 were performed at an intracellular ATP concentration of 1 mM. Omitting ATP accelerated the rate of opening of WT channels and this effect was not due to differences in Mg$^{2+}$ chelation (results not shown). Replacing ATP with AMP accelerated the rate of opening even more markedly and significantly for WT and G715E channels but not for the mixed WT+G715E channels. Fig. 4A compares the kinetics of activation at –130 mV and deactivation at 30 mV for the three channel types in AMP. Both activation and deactivation occurred with apparent rates in the ranking order WT > G715E > WT+G715E. In Fig. 4B and C average time constants are compared in ATP and AMP experiments. Both slow and fast time constants were significantly decreased by AMP replacement of intracellular ATP in WT and G715E channels. The small decrease observed for WT and G715 co-expression did not reach statistical significance. Similar results (not shown) established that the
deactivation by the post-pulse to 30 mV was similarly affected by AMP replacement. There was no significant effect on the steady-state voltage-dependence (not shown).
Discussion

In this paper we present a functional analysis of the consequences of IGE-associated mutations of the ClC-2 Cl⁻ channel on its function. Our results are in marked contrast to those reported previously by Haug et al. (18) and suggest that the pathophysiological mechanisms proposed by these authors to account for the phenotype need to be revised.

The M200fsX231 mutant of ClC-2 is predicted to produce a severely truncated protein. Possible fates for this putative protein include its elimination by quality control checkpoints during its biogenesis and trafficking, remaining in intracellular compartments, and reaching the plasma membrane. Haug et al. (18) studied this point by transfection of fluorescence-labeled fusion proteins and confocal microscopy. Their interpretation of the fluorescence data was that the mutant proteins reached the plasma membrane. It is difficult to distinguish, however, true membrane localization with the presence of intracellular fluorescence near the plasma membrane. We have used here a double-labeling approach with ClC-2-GFP channel fusion proteins and di-8-ANEPPS, a specific marker for the plasma membrane. The study of WT ClC-2 reveals a complex localization pattern. There is abundant intracellular protein in vesicular structures. In addition there is plasma membrane labeling, revealed by superposition with di-8-ANEPPS, which suggests that ClC-2 is localized in discrete membrane areas. The distribution of ClC-2-GFP protein in transiently transfected HEK-293 cells is similar to that seen by immunofluorescence in COS7 fibroblasts that express ClC-2 endogenously. The distribution in COS7 cells was seen to correspond to plasma membrane, early
endosome and perinuclear localization (8). This type of distribution is consistent with the results observed here. In addition preliminary experiments (not shown) also identify a vesicular distribution of ClC-2-GFP in an early endosomal compartment.

By contrast with the subcellular distribution of WT-GFP, that of the M200fsX231-GFP fusion protein was entirely intracellular and there was no obvious superposition with di-8-ANEPPS fluorescence. It is possible that a dominant negative effect might arise by M200fsX231 altering the trafficking of WT proteins preventing their access to the membrane. Functional experiments, however, reveal no hint of such an effect, as co-expression of WT-GFP and M200fsX231 produced robust currents in our hands. It is interesting to note that truncation mutations of ClC-1, also a dimeric chloride channel, have always been found to be associated with recessive myotonia (22). The analysis of the alternatively spliced protein Δ74-117 leads to very similar conclusions. Co-transfection of the mutant cDNA together with the WT channel cDNA produced sizable currents, with no evidence of a dominant negative effect of the mutant. In addition, transfection of HEK-293 cells with cDNA encoding a Δ74-117-GFP fusion protein gave a purely intracellular, reticular in appearance, distribution for the GFP fluorescence.

The experiments of co-expression of M200fsX231 and Δ74-117 mutants with WT channels were performed with the same amount of total cDNA as those of expression of WT alone. This implies that the amount of WT cDNA was halved. The currents obtained, although lower than those seen with twice the amount of WT cDNA, was higher than
50%. We believe that this is caused by the lack of linearity between amount of cDNA used in transfection and activity of expressed protein (unpublished observations).

A puzzling finding concerning the IVS2-14del11 mutant is that the alternatively spliced mRNA that was predicted by Haug et al. to arise from its expression has been found both in patients and healthy controls (18). Using a minigene approach, we find that regardless of the presence or absence of the deletion, an exon 3-skipped mRNA is produced, in addition to the normally spliced mRNA. The same result was obtained in N2a neuroblastoma cells, GT1-7 mouse hypothalamic neurons and HEK-293 cells. Unlike the result obtained with human samples, we find no difference in the proportion of exon-skipped to normally spliced mRNA in any of the cell lines transfected with minigenes mimicking mutated or WT genes. It is not possible to state whether the results obtained in experiments with minigenes are representative of the splicing mechanisms taking place \textit{in vivo}. The fact that the correct splicing did take place, however, argues for similar mechanisms applying to the minigene products in the cell lines as with the RNA arising from the entire gene. On this basis we would have to conclude that there is no difference in the way that mutated and WT gene products are treated by the cellular splicing machinery. We have no simple explanation for the discrepancy between previous results and our minigene experiments. Our results suggest, nevertheless, that there will be no difference in ClC-2 channel expression between normal individuals and those carrying mutation IVS2-14del11.
A further question that applies to both M200fsX231 and Δ74-117 is whether the severely truncated or deleted proteins produced, survive the quality control mechanisms at the endoplasmic reticulum and beyond. This quality control mechanisms are known to deal with many conditions that result in altered, non-native protein conformation leading to retention in the endoplasmic reticulum followed by translocation to the cytosol and degradation (44).

The third mutation associated with IGE is a missense mutation, G715E, affecting an amino acid located in the long intracellular carboxy-terminus of the channel between the two CBS domains. Haug et al. (18) have proposed that this mutation provokes a change in the channel gating making it less sensitive to internal chloride concentration. This would imply that the activity of the channel at very low intracellular Cl⁻ is higher in the mutant than in non-mutated channels. We have examined this point, but our results show no differences regarding voltage-dependence of gating and kinetic parameters between WT and G715E channels. Moreover, we could not detect any difference in Cl⁻-dependence.

It might be interesting to point out that a C-terminus endoplasmic reticulum export signal (FCYENE) was used in the experiments reported by Haug et al. (18). This export signal has been reported to profoundly alter the steady-state distribution of channels in the plasma membrane increasing the number of proteins on the cell surface (26). We cannot discard that this difference might be at the source of the discrepancy between our results and those reported before (18).
Two different gating processes - a fast and a slow gate - have been proposed for the voltage-dependent opening of ClC-2 (47). These are analogous to those first proposed for ClC-0, the voltage-dependent Cl⁻ channel of *Torpedo* electroplax (31). Slow and fast gating of ClC-2 are promoted by hyperpolarization. Fast gating depends additionally on intracellular Cl⁻, an effect that is reflected in a shift of the $V_{0.5}$ to more a depolarized potential with increasing intracellular concentration. This intracellular Cl⁻-dependence of gating is a property of the pore, more specifically of competition between Cl⁻ and a glutamic acid residue side chain for an outermost Cl⁻-binding site in the selectivity filter (11). Neutralization of this residue by mutation in ClC-2, abolishes all Cl⁻-dependence of the channel (32). It is, therefore, difficult to envisage how a point mutation far from the pore might alter the sensitivity to intracellular Cl⁻. The slow gate, however, seems to be independent of intracellular chloride (32). Regarding this point there is a recent interesting suggestion that CBS domains could play a functional role in the common slow gate (12). Two CBS domains are present in the carboxy terminus of all eukaryotic ClC channel subunits. Point mutations within them cause several hereditary diseases in humans. Examples in the CIC family are CIC-1-related myotonia, Dent’s disease caused by CIC-5 mutations and Bartter’s disease arising by mutation of CIC-Kb (24; 25; 39). Although the physiological role of CBS domains is still unknown, truncations removing parts of the distal CBS domain of ClC channels abolished functional expression in heterologous systems (20; 36).
Interestingly, Scott et al. (37) recently demonstrated that the isolated CBS domain pair from ClC-2 binds ATP and that the affinity decreases one order of magnitude with introduction of G715E mutation. We tested if there was an electrophysiological correlate of this effect. Our results demonstrate that adenine nucleotides are able to modify the kinetics of the ClC-2 channel. The typical slow activation of the channel is accelerated when intracellular AMP replaces ATP. Both the slow and fast voltage-dependent opening processes are significantly accelerated by AMP replacement. This was observed in the WT and to a lesser degree in the G715E mutant. However when WT and G715E were co-expressed, the effect of AMP for ATP replacement on opening kinetics was smaller and did not reach statistical significance. To explain this unexpected result we would have to assume that the dimer made by the association of one WT and one G715E channel would be completely unresponsive to nucleotide replacement, thus producing a dominant negative effect. As the contribution of the heterodimer to function is expected to be 50%, this would account for the result obtained.

What is the physiological meaning of this finding? It is tempting to speculate that the channel, through its CBS domains, is sensing changes in the energetic state of the cell by detection of AMP level. The AMP level is thought to be a key signal to regulate various enzymes under metabolic stress (17). The best studied of these is AMPK, which possesses four CBS domains in tandem in its γ subunit. The enzyme is allosterically regulated by AMP, which binds to CBS domain pairs. Human disease-causing mutations impair AMP binding (1). Fusion protein studies suggest that each CBS pair forms a binding site and that there is strong positive cooperativity between them (37). It has been
proposed that CBS domains in ClC-2 might fulfill a similar role and that mutation G715E
impairs nucleotide binding (37). The effects of AMP on the kinetics of opening of ClC-2
observed here might be related to this postulated effect. As G715 is not in, but between
CBS domains, perhaps the mutation alters the three-dimensional structure necessary for
the postulated CBS1/CBS2 interaction (12). This might explain the decreased AMP effect
seen in the mutant. To explain the even feebler effect of AMP for ATP replacement in the
mixed WT and G715E experiment, we have speculated that the heterodimer is
unresponsive to intracellular nucleotides. We have attempted to confirm this
interpretation by using WT-WT and WT-G715E concatemers constructed as described
previously for ClC-2 (46). Surprisingly, neither concatemer was responsive to nucleotide
replacement (results not shown). This might be expected if an interaction between the
CBS pairs from each monomer were necessary for optimal nucleotide binding/sensing.
The concatemerization process, that tethers the C-terminus of one protein to a short N-
terminus of another, might destroy this putative interaction.

What would be the consequences of mutations of ClC-2 associated with IGE? This point
can only be speculated upon, given the fact that the function of normal ClC-2 in the brain
has not been elucidated. In neurons ClC-2 is postulated to prevent paradoxical GABAAR-
mediated excitatory actions (42) whilst a role in extracellular electrolyte homeostasis is a
proposed function in astrocytes (38). A dysfunction of either of these processes could
lead to disturbances in neuronal excitability and could explain the epileptic phenotype.
However, lack of epilepsy in the knock out ClC-2 mouse (5) makes it difficult to
postulate a straightforward explanation for the relationship between the mutations and a
pathophysiological mechanism. Haug et al. (18) have proposed dominant negative effects for mutations M200fsX231 and Δ74-117 of ClC-2. Although we find these two mutants to lack function, we do not find any evidence for dominant negative effects in co-transfection experiments. In addition, our minigene experiments suggest that the Δ74-117 product would arise equally well from a WT or a IVS2-14del11 gene. We have no explanation for this discrepancy. On the basis of our results, we would have to postulate a mechanism of haploinsufficiency to explain the phenotype through a decrease in the number of copies of active channels in the case the mutation producing M200fsX231. Our findings with the G715E mutation highlight the potential importance of the C-terminus of the protein, including the CBS domains, in an unexpected mechanism of channel regulation. A failure in the G715E mutant channel to respond with accelerated kinetics to a situation of cellular stress depleting ATP and elevating AMP could be a potential pathophysiologic mechanism. These situations of high energy consumption might arise in conditions leading to hyperexcitability that might become pathological in individuals carrying the mutation. The effect observed, however, is far from being deleterious and we are not able to propose a solid pathophysiologic mechanism for a possible action. A more detailed delineation of the function played by the kinetic change brought about by AMP in ClC-2 will be required for a full interpretation of these results. It must be pointed out that G715E mutation has only been found in a small family and with only one sib not carrying the mutation and being phenotypically normal. More genetic data will be needed to establish whether this is a genuine mutation or a benign polymorphism.
Finally, our results indicate that there is no simple relationship between ClC-2 mutations and the IGE pathophysiology. This might be due to the fact that we still do not know what the function of the channel is in the normal brain. On the other hand, the analysis of the pathophysiology of the disease-associated mutations is now being complicated by the increasing realization of the influence of modifier genes in the phenotype of human inherited disease. Although some traits are still recognized to be inherited in a monogenic fashion, exceptions to this rule are now being reported (2). The difficulty in generating a genotype-phenotype relation in the case of alterations in CLCN2 might be a consequence of such complexity. The true physiological and cellular nature of the defect might have to await a deeper understanding of the genetic background upon which the defects are expressed.
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23. Kaila K, Lamsa K, Smirnov S, Taira T and Voipio J. Long-lasting GABA-mediated depolarization evoked by high-frequency stimulation in pyramidal


40. **Smith RL, Clayton GH, Wilcox CL, Escudero KW and Staley KJ.** Differential expression of an inwardly rectifying chloride conductance in rat brain neurons: a


Figure legends

Fig. 1. *Functional analysis of the co-expressed WT and M200fsX231 or Δ74-117 mutant channels.* A and C: Steady-state activation as a function of voltage is plotted for WT and WT+mutant co-expression. The line is the result of fitting equation (2) to the WT data. The fits to data for the WT+mutant co-transfection did not differ significantly from that of the corresponding WT experiments. Mean values ± SEM for slope factor and $V_{0.5}$ (both in mV) were as follows: WT-GFP, $-24 \pm 1$ and $-104 \pm 3$ (n=11); WT-GFP+M200fsX231, $-25 \pm 1$ and $-110 \pm 3$ (n=9); WT, $-23 \pm 1$, and $-108 \pm 4$ (n=11); WT+Δ74-117, $-27 \pm 1$ and $-113 \pm 3$ (n=13). B and D: $G_{\text{max}}$ of WT and of co-transfected WT+mutant. $G_{\text{max}}$ corresponds to the maximal conductance at full activation, extrapolated according to equation (2). Points correspond to individual values and lines connect average values. E: Effect of an 11 bp-deletion in *CLCN2* intron 2 on the mRNA splice processing. The diagram shows the minigene constructs containing *CLCN2* exons 2, 3 and 4 (boxes) and the corresponding introns (straight lines). The splice patterns are represented as diagonal lines resulting in 495 bp and 365 bp bands. The filled triangle represents the 11 bp-deletion in intron 2. The agarose gel shows the results of RT-PCR from N2a cells transfected with WT and intron2Δ11bp deletion. Lane 1: water control. Lanes 2 and 5: WT and intron2Δ11bp deletion, controls without reverse transcriptase. Lanes 3 and 6 WT and exon2 11bp deletion PCR products. Lane 4: Standard 100 bp ladder (Invitrogen).
**Fig. 2.** M200fsX231 and Δ74-117 mutants do not reach the plasma membrane. HEK-293 cells were transiently transfected with fusion proteins WT-GFP (A-C), M200fsX231-GFP (D-F) or Δ74-117-GFP (G-I) and analysed by confocal microscopy. The sub-cellular distribution of the different fusion proteins is shown in A, D and G. To label the plasma membrane the transfected cells were incubated with di-8-ANEPPS shown in red in B, E and H. An overlay of both fluorophores is shown in C, F and I. Bar is 5 μm.

**Fig. 3.** Gating properties of the G715E mutant. Steady-state activation as a function of voltage is plotted for WT and the G715E mutant. Results in A and B were obtained using 35 and 10 mM [Cl\(-\)]\(_{i}\) respectively. The lines are the result of fitting equation (2) to the WT data. The V\(_{0.5}\) values (mean ± SEM, number of experiments in brackets) were as follows: [Cl\(-\)], 35 mM, WT –108 ± 4 mV (11) and G715E –104 ± 3 (6); [Cl\(-\)], 10 mM, WT –128 ± 4 mV (5) and G715E –125 ± 3 (7). Equation (1) was adjusted to the time course of current activation in experiments at 35 mM [Cl\(-\)]\(_{i}\). The voltage-dependence of the slow (τ\(_s\)) and fast (τ\(_f\)) time constants is shown in C and fractional amplitudes for the slow, fast and instant terms, A\(_s\), A\(_f\) and A\(_0\) respectively, are shown in D. WT, circles; G715E, triangles. Means ± SEM of 11 and 6 separate experiments for WT and G715E.

**Fig. 4.** Effect of intracellular AMP and ATP on ClC-2 and G715E-mediated currents.
A: Representative current traces, elicited from a V\(_h\) of -10 mV in response to a pulse to –130 mV followed by a pulse to 30 mV, for WT, G715E and WT+G715E, in the presence of 2 mM intracellular AMP. For illustration proposes, the beginnings of the tail currents
at 30 mV were set at the same time. The currents have been normalized to the maximal current during the -130 mV portion of the trace for WT. B: Activation time constants obtained in presence of 1mM intracellular ATP or 2 intracellular mM AMP. The time course of activation during the –130 mV pulse was adjusted by equation (1). Slow (left hand panel) and fast (right) time constants are shown. The p values for t-test of the differences between Tau slow values with ATP vs AMP were as follows: WT 0.0002, G715E 0.0045 and WT+G715E 0.287. Corresponding p values for Tau fast differences were: WT 0.0002, G715E 0.033 and WT+G715E 0.358.
FIG. 1
WT-GFP

M200fsX231-GFP

Δ74-117-GFP

FIG. 2
FIG. 3
FIG. 4