Alterations in the Cytoplasmic Domain of CLCN2 Result in Altered Gating Kinetics

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Key Words

CLCN2 mutations • Anion channel • CBS domain • Electrophysiology • Cell swelling • Gating kinetics

Abstract

Mutations in the human ClC-2 Cl⁻ channel have been described to influence its function dramatically. To test for naturally occurring gene variants in a human population and their functionality, all 24 *CLCN2* exons from a Central African population were sequenced. Six single amino acid exchanges in the intracellular N-terminus (P48R, R68H), in the pore domain (G199A), or in the intracellular C-terminus (R646Q, R725W, R747H) were identified at low frequency. Heterologous expression of these polymorphisms in *Xenopus laevis* oocytes demonstrated their functional significance as determined by two-electrode voltage-clamp. The polymorphisms R68H, R725W, and R747H exhibited faster voltage-stimulated gating as compared to the wild type channel, resulting in higher steady state currents of R725W. Probably due to decreased surface expression P48R, R68H, and R646Q mutants generated lower currents than the wild type channels. The inward currents of the mutated channels R725W, R747H, and G199A failed to

increase during hypotonic swelling, a defect paralleled by impaired swelling-accelerated voltage-gating in one mutant (G199A). In conclusion, the Africans' gene pool comprises *CLCN2* gene variants in the N-terminus, the C-terminus or the pore domain that affect surface expression and voltage- or cell-swelling-stimulated channel gating.

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Introduction

The human *CLCN2* gene encoding the inwardly rectifying chloride channel ClC-2 is located on chromosome 3q26. It comprises 24 exons which were first sequenced by Thiemann et al. in 1992 [1, 2]. The recently identified crystal structure of a bacterial ClC orthologue defines the molecular structure consisting of 18 α -helices that exhibit a complex topology and form a homodimer interacting at a broad interface [3]. Each subunit forms its own pore (protopore) and selectivity filter. Several splice variants of ClC-2 have been

described [2, 4-6] showing altered channel function [2].

The halide selectivity sequence of CIC-2 is $\text{Cl}^- \geq \text{Br}^- \geq \text{I}^-$ [1]. In *Xenopus* oocytes [1, 7, 8] as well as in transfected mammalian or insect cells [9] CIC-2 currents activate slowly upon hyperpolarization. Moreover, CIC-2 channels are reversibly activated by hypotonicity-induced cell swelling [7, 9] and lowering of the extracellular pH [8-10]. In addition, CIC-2 activity depends on the intracellular Cl^- concentration [11]. CIC-2 mRNA has been detected in every tissue and cell line examined [1]. Accordingly, hyperpolarization-activated CIC-2-like Cl^- currents have been observed in various tissues and cells.

According to its localization to apical membranes of epithelial cells CIC-2 might contribute to transepithelial Cl^- and fluid transport and to gastric acidification [12]. CIC-2 has also been detected in fetal lung and collecting duct and a function of CIC-2 in branching morphogenesis of these organs has been suggested [5, 13, 14]. In addition, the CIC-2 orthologue of *C. elegans*, CLH-3, has been suggested to function in heterologous cell-cell interactions, intercellular communication, and regulation of cell cycle-dependent processes [15, 16]. Moreover, localization of CIC-2 in rat brain close to inhibitory synapses suggests a CIC-2 function in GABA-ergic transmission [17]. Loss of CIC-2 might result in neuronal hyperexcitability. A susceptibility locus for common idiopathic generalized epilepsy has been mapped to human chromosome 3q26 [18], which is close to the human *CLCN2* gene [19]. Accordingly, three different heterozygous mutations in the human *CLCN2* associate with idiopathic generalized epilepsies: These mutations result in (i) a premature stop codon (M200fsX231), (ii) an atypical splicing (del74-117) and (iii) a single amino-acid substitution (G715E). All mutations produce functional alterations that provide distinct explanations for their pathogenic phenotypes [20]. In contrast to the proposed CIC-2 functions in humans, the *clcn2*^{-/-} mice do not suffer from spontaneous seizures, do not show any defect in lung or kidney development and display normal gastric acidification. The *clcn2*^{-/-} mice exhibit a degeneration of photoreceptors and male germ cells leading to the total loss of both cell types in the adult animals [21].

Most recent evidence points to some functional significance of CIC-2 for the blood stage of the malaria infection. CIC-2-mediated anion currents have been demonstrated in *Plasmodium*-infected but not in non-infected human and mouse erythrocytes. CIC-2 contributes to the regulatory cell volume decrease (RVD) of the volume-expanding host erythrocyte because inhibition of CIC-2 by ZnCl_2 leads to a volume increase of *Plasmodium*-

infected *clcn2*^{+/+} erythrocytes but not of infected *clcn2*^{-/-} erythrocytes or non-infected *clcn2*^{+/+} and *clcn2*^{-/-} erythrocytes [22]. The present study aimed to identify polymorphisms in the human *CLCN2* gene which might affect the CIC-2 channel function. Since African populations are generally more diverse in their genetic repertoire we focussed our search for variants in a population living in Central Africa, Gabon.

Materials and Methods

Patients

From a population living in Central Africa, Gabon, 24 exons of *CLCN2* in Africans (n= 35) were sequenced. Patient samples were either from a combination therapy trial [23, 24] or from a longitudinal survey to evaluate immunological effects of malaria [25, 26]. Ethical clearance for these trials was given by the ethics committee of the International Foundation for the Albert Schweitzer Hospital.

Isolation of DNA

DNA was purified from 200 μl of blood from Africans using the Qiagen blood kit (Qiagen, Hilden, Germany). Exons were amplified either separately or in combination with neighboring exons. Primer sequences for DNA analyses of *CLCN2* exons are provided in Tab 1. Primers were purchased from Operon, Cologne, Germany. The PCR products were subjected to electrophoresis on a 1% agarose gel in 1xTBE buffer (100 mM Tris, 86 mM borat, 2 mM EDTA) and were purified with the E.Z.N.A. Cycle pure kit (peqlab, Erlangen, Germany) following the manufacturer's instructions. DNA sequencing was carried out using the BigDye Terminator kit (version 1.1; Applied-Biosystems, Foster City, USA). The cycle PCR conditions were as follows: 25 cycles were carried out at an annealing temperature of 50°C for 30 sec, an extension temperature of 60°C for 4 min, and a denaturing temperature of 96°C for 15 sec. Cycle-PCR was performed by using between 0.5 μl and 4 μl of the PCR products according to their DNA concentration. The reaction volume contained 1 μl Big Dye™, 1 μM primer, 2 μl 5 x reaction buffer (Qiagen). H_2O was added to a final volume of 10 μl . Fragments were analysed using the ABI Genetic Analyzer 3100 (Applied-Biosystems, Foster City, CA). Sequence comparison and detection of mutations was done using SeqScape (Applied-Biosystems, Foster City, CA).

Two-electrode voltage-clamp

Xenopus laevis oocytes were singularized by collagenase treatment and mechanical forces which digested/removed most of the vitelline coat. Singularized oocytes increased cell volume when superfused with hypotonic medium (not shown). Human CIC-2 cRNA [20] (2.76 ng) or a volume of water equal to the volume of RNA solution was injected in oocytes and currents were recorded by two-electrode voltage-clamp 2 days after injection as has been described in detail elsewhere [27].

	forward	reverse	seq-primer
Exon1	940F: ggagccgagtcaggacaga	1372R: tgctgagactgggcccagg	
Exon2-3	3298F: ttccactcagccatgacct	3873R: ctccatgtctagacgagtgtggc	3583R: ccccatcagcagctctaag
Exon4-8	4097F: tatcagcgtggctccactggc	5167R: ccctacctggcaccatctcg	4644R2: gcttgcatatgcacaaaagg 4544F2: agtcctcctggtggaaga
Exon9	5167F: cgagatgggtccaggtaggg	5315R: tcctctctgataccccagc	
Exon10	5315F: gctgggggtatcagagagga	5548R: cctgacctactgtggcccttat	
Exon11-12	6630F: ctcagccctgagatatgcga	7163R: agggtccttagaagggaagct	
Exon13-14	7412F: ccacctgatcagcaggttagact	8436R: atctaggatgccctctgccctt	8008R: caccagagtctcggacctgt
Exon15	8107F: ttgtggagggtcttctgggac	8436R: atctaggatgccctctgccctt	
Exon16-18	8583F: atggatgggctctgggcctt	9521R: agggctgggcagggactcta	8620F: atggcaatgctctgggcacct 9500R: tggatctcagtagcttggctgagg
Exon19-22	9607F: ctttctgtgtctctcccaaa	10530R: gaccaagatccccactgtgtcc	
Exon23-24	15395F: tggcctgtcatttagagctg forward	15933R: atgcacattctgggctgac reverse	seq-primer

Table 1. Primer sequences for DNA analyses of *CLCN2* exons

Oocytes were first recorded in isotonic ND 96 solution containing (in mM) 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 Tris-HEPES, pH 7.4. To study the dependence of ClC-2 currents on oocyte volume cells were initially recorded with isotonic ND 48 + sucrose solution (in mM) 48 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 96 sucrose, 5 Tris-HEPES, pH 7.4, and then osmotically swollen in hypotonic ND48 solution (in mM) 48 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 Tris-HEPES, pH 7.4.

Modeling

The X-ray dimer-CBS domains structure of the inosine monophosphate dehydrogenase (IMPDH [28]) from the bacteria *S. pyrogens* were retrieved from the Protein Data Bank. A three dimensional structural model of the CBS-1 domain of ClC-2 was constructed based on homology to IMPDH-CBS using the solved crystal structures of corresponding domains using a similar approach as reported by Estevez et al., 2004. The alignment of ClC-2 and mammalian homologues is shown in Fig. 7A. The ClC-2 models were generated using SWISS-MODEL (<http://www.expasy.org/swissmod/SWISS-MODEL.html>) and energy optimized using Gromos96 in default settings within the Swiss-PdbViewer [29]. In the model with the lowest energy we calculated putative h-bonds with distances of 1.2 to 2.76. Subsequently, we performed the virtual mutagenesis of

Arg646 to Gln646 and energy optimized the resulting model using the same force field constraints. In the R646Q-model with the lowest energy we again calculated putative h-bonds with the same distances.

Results

Mutational analysis

The DNA sequence of all 24 exons from 35 individuals was determined. A total of 11 fragments were amplified by PCR harboring all exons. DNA sequencing was done with the same primers used to generate the PCR products, or with primers internally to those (Tab. 1). With the exons neighboring or intermediate intron sequences were analyzed as well. The coding region consists of 2694 bp. From adjacent intron sequences around 1500 base pairs were analyzed. In the introns we found 10 mutations, none of which affected intron/exon boundaries. Seven mutations in the exons led to amino

Table 2. Polymorphic positions in the CLCN2 gene. Given are the 25 flanking bases 3' and 5' of the SNP. The numbering is according to the accession number NC_000003; numbering in *italic* represents the chromosomal location according to GenBank entry from the 3rd of March 2006; localisation in the intron and affected codons in the coding sequence are indicated. *, **, and *** had been submitted to the SNP data base with the accession numbers rs2228291, rs3749222, and rs9820367, respectively.

Amino acid	Major allele	Minor allele
P48R	0.985	0.015
R68H	0.944	0.056
I170I	0.985	0.015
G199A	0.985	0.015
I302I	0.556	0.444
N542N	0.985	0.015
R646Q	0.985	0.015
S668T	0.765	0.235
R725W	0.958	0.042
R747H	0.985	0.015

Table 3. Frequency of exon mutations in African individuals

acid exchanges, 3 represented synonymous mutations (Tab. 2). Eight out of 10 polymorphisms had an allele frequency of less than 6% (Tab. 3). Two polymorphisms had a higher frequency. The amino acid serine at position 668 was changed to threonine in 14 individuals (two were homozygous, 12 were heterozygous at this position).

Position	Flanking sequences	Affected amino acid/localisation
2428	CGGATTCGCCTGGGAGGGCCTGAAC (c/g)	P48R
<i>185559534</i>	CTGGAAGGTCCCCCTTCCTCTCGG	
2488	GAGCTCTTGAATATGGACGGAGCC (g/a)	R68H
<i>185559474</i>	TTGCGCCCGATGCCCGGTGAGAAC	
2560	AGCAGAGGCCATTAGAGCTGCTGAT (g/c)	intron
<i>185559382</i>	GGGGAGAGGGGACAGTTGAGGGGGC	
3310	CCCTCAGGCTGTCGGTATGTAGAA (g/a)	intron
<i>185558652</i>	AGAAGGGGAGGGCAGAGGCGGGACC	
3368	CTGGCATCCCTGAGATGAAGACCAT (c/t)	I170I
<i>185558549</i>	TTGCGGGGAGTGGTGTCTGAAAGAAT	
3499	GGGCTGACCTGCGCCCTAGGCAGCG (g/c)	G199A
<i>185558463</i>	GATGCCGCTTGGAAGAGGTAACCT	
4221*	TGCTGTCTTCCCTTTAGAGACTAT (t/c)	I302I
<i>185557741</i>	ACAGCCCTCTTCAAAACCCGATTCC	
5917	CATGGCCTCTCCCTTCATCCTGTC (g/a)	intron
<i>185556045</i>	GTTTCCCAGCCTGAGGCACCCTGTT	
6636**	CCTTCCTCAGCCTCTGGCCACCCCC (c/g)	intron
<i>185555326</i>	CAGCCAACCCTCTGCCCAAGAGCGG	
6636/7	CCTTCCTCAGCCTCTGGCCACCCCC (cc/aa)	intron
<i>185555325/6</i>	AGCCAACCCTCTGCCCAAGAGCGGG	
6809	TCCACTGCCTCTTCTCTTCCACC (c/t)	intron
<i>185555153</i>	CCCTCCCTGAAGGAGCAGCATTGG	
8140	GCCCAGCTGAGCCCAGCCCGCCGGC (g/a)	R646Q
<i>185553823</i>	GCAGCACATGCAGGAGCGCAGAGCC	
8205***	CTATCTGATCAGGAGGGTCCCCCTA (c/g)	S668T
<i>185553757</i>	CCCTGAGGCTTCTGTCTGCTTCCAG	
8249	CTTCCAGGTGAGGGGAAAAGCCACA (a/c)	intron
<i>185553713</i>	ACGCTTCCTAAATGTCATTGTGTAG	
8699	CGCAGAGTCGGCAGGCATCGCCCTC (c/t)	R725W
<i>185553263</i>	GGAGCCTCTTCTGTGGCAGTCCACC	
9006	AAGAAGTTGGAATCCTGTGAGAAGC (g/a)	R747H
<i>185552956</i>	CAAGCTGAAGCGTGTCCGAATCTCC	
9045	GTCCGAATCTCCCTGGCAGTAAGTA (t/g)	intron
<i>185552917</i>	GCCTGCTCTTCTAGCATCTCGGCGG	
9273	GCTGCTGTCCCCATGGGAGGGACCA (c/a)	intron
<i>185552689</i>	GGGGTTGGAAAAAGGGCAGGCCAGC	
9330	GAGGTGAGGTGACGCTACAGGCACCT (t/g)	intron
<i>185552632</i>	TCTCCCTAAACCTCATAGTCTCTTC	

A second highly polymorphic but synonymous exchange was at amino acid 302. 23 individuals had a base exchange from cytosine to thymine (nine were homozygous, 14 were heterozygous for thymine). These two positions were not in linkage disequilibrium with each other. Since the frequent polymorphisms 668S and 668T have been

Fig. 1. Model of the CLC protein (modified from [20]) showing the positions of the analyzed polymorphisms.

characterized in previous work we focused in this study on the less frequent mutations. The amino acid exchanges P48R, R68H, G199A, N542N, R646Q, R725W, and R747H are situated at the cytosolic N-terminus, in the pore domain, or the cytosolic C-terminus of the CLC-2 channel protein as shown in Fig. 1.

Two-electrode voltage-clamp recording

To test for the functional significance of the identified polymorphisms in *CLCN2*, wild type and mutated CLC-2 mRNA (cRNA) was injected in *Xenopus laevis* oocytes and the CLC-2 channel activity determined by two-electrode voltage clamp. Fig. 2A, upper panel and Fig. 2B show the steady state currents of water-injected and CLC-2 wild type mRNA-injected oocytes as recorded in isotonic ND96 bath solution. Heterologously expressed CLC-2 exhibited an inwardly rectifying current-voltage relationship with a current reversal potential in the range of -20 to -30 mV (Fig. 2B). Two mutants (G199A and R747H) had current-voltage relationships identical to the

wild type channel (Fig. 2C and D). Three mutants (P48R, R68H, and R646Q) generated lower currents which resulted in a significantly lower inward conductance as compared to the wild type control (Fig. 2A, lower left and Fig. 2C and D). The mutant R725W, in contrast, generated significantly larger inward currents than wild type CLC-2 at high negative voltages (Fig. 2A, lower right and Fig. 2C).

Hyperpolarization-stimulated gating of CLC-2 channels occurs with a fast ($T_{\text{act-fast}}$) and a slow time constant ($T_{\text{act-slow}}$) probably reflecting opening of the protopores and removal of an inactivation complex, which regulates gating of both individual protopores, respectively [10]. In the present study, we analyzed $T_{\text{act-slow}}$ and the deactivation kinetics (T_{deact}) upon hyperpolarization from the holding potential to -140 mV test voltage and repolarization from -140 mV to +50 mV. Using this protocol, CLC-2 wild type channels activated and deactivated with a time constant of about 0.5 s ($T_{\text{act-slow}}$) and 0.8 s (T_{deact}), respectively (Fig. 3A, C, left and Fig.

Fig. 2. Voltage-gated Cl⁻ currents generated by heterologously expressed CIC-2 variants as analyzed by two-electrode voltage-clamp. **A.** Current traces of a H₂O-injected oocyte (upper left), a CIC-2 wild type mRNA- (upper right) and two (P48R, R725W) CIC-2 mutant mRNA-injected oocytes (lower left and right). The currents were recorded 2d after injection. The inset shows the applied pulse protocol. **B.** Current-voltage relationships of H₂O-injected (closed triangles) and CIC-2 wild type mRNA-injected oocytes (open circles) recorded as in (A; means $\pm$ SE; n = 6-14). The steady state current at the end of the 1st voltage sweep has been analyzed. **C.** Normalized current-voltage relationships of wild type mRNA-injected (open circles) and mutated CIC-2 mRNA-injected oocytes (closed triangles). The amino acid exchange of the mutants is indicated. The currents of the mutants were determined in at least three different oocyte preparations. For each oocyte preparation oocytes with wild type mRNA were recorded in parallel (open circles) and currents were normalized to the -140 mV value of the corresponding wild type CIC-2 current (arithmetic means $\pm$ SE; n = 12-72; *, **, p $\leq$ 0.05, 0.01; ANOVA). **D.** Normalized slope conductance as calculated from (C) for the inward current by linear regression between -60 and -140 mV. Conductances were normalized to those of the wild type CIC-2. Data are arithmetic means $\pm$ SE; n = 12-72. **, and ***: indicate significant difference from 1.0 (p $\leq$ 0.05, 0.01, and 0.001, two-tailed t-test, respectively).

3B, D). P48R, G199A, und R646Q exhibited identical gating kinetics (Fig. 3B, D). In contrast, the mutant R68H, R725W, and R747H showed significantly faster activation (Fig. 3B) while deactivation was unaltered as compared to the wild type channel. Hyperpolarization-stimulated activation was similar in wild type CIC-2 and all mutant channels had a half-maximal voltage of about -80 mV

(Fig. 3E, F). In summary, all mutant channels except G199A differed either by absolute currents or by their gating kinetics from the wild type channel.

Hypotonic swelling of the oocytes by decreasing the bath osmolarity to 50% stimulated an increase in wild type CIC-2-generated inward currents (Fig. 4A, B). Similarly, current increase was observed for P48R-,

Fig. 3. Voltage dependence and kinetics of the CIC-2 channel variants. *A.* Slowly activating currents evoked by a voltage step from -60 mV holding potential to -140 mV voltage. Current traces of a CIC-2 wild type mRNA- (left) and a R725W-mutant mRNA-injected oocyte (right) are depicted. The hyperpolarization-stimulated time-dependently activating current was fitted mono-exponentially. The fitted curve is superimposed (bold line). *B.* Time constant of activation (T_{act} ; arithmetic means $\pm$ SE; $n \leq 12-72$; determined as in *A*) of wild type and mutant CIC-2 channels (*: $p < 0.05$; **: $p \leq 0.01$; ANOVA). *C.* Deactivating currents of a wild type mRNA- (left) and a R646Q mutant CIC-2 mRNA-injected oocyte upon depolarization from -140 mV test potential to +50 mV voltage. Currents were fitted mono-exponentially. The fitted curve is superimposed (bold line). *D.* Time constant of de-activation (T_{deact} ; arithmetic means $\pm$ SE; $n = 12-72$; determined as in *C*) of wild type and mutant CIC-2 channels. *E.* Dependence of wild type CIC-2 channels on the voltage. The CIC-2-generated conductance was determined for the steady state current, plotted against the test potential and fitted by the Boltzmann equation (bold line). *F.* Mean half-maximal voltage (arithmetic means $\pm$ SE; $n = 11-73$) of wild type and mutant CIC-2 channels as determined by a Boltzmann fit.

R68H-, and R646Q- mutant channels (Fig. 4C). However, swelling failed to significantly stimulate the inward currents in G199A-, R725-, and R747H-mutant CIC-2-injected oocytes (Fig. 4D). In addition, no increase of inward currents was observed during hypotonic swelling of water-injected oocytes ($n = 6$; Fig. 5A, upper line). In wild type CIC-2-injected oocytes (but not in water-injected oocytes; Fig. 5A) cell swelling increased the outward current, which inactivated time-dependently (Fig. 4A). Similarly, cell swelling stimulated outward currents in all mutant CIC-2-injected oocytes as evident from the current-voltage relationships in Fig. 4C and the calculated conductances in Fig. 4D (upper bars). To confirm that these outward

currents are indeed generated by CIC-2 and not by endogenous oocyte channels [30], the CIC-2 channel inhibitor $ZnCl_2$ (1 mM) was applied to water-, wild type- and G199A mutant-injected oocytes recorded in isotonic and hypotonic solution (Fig. 5A). In isotonic bath solution, $ZnCl_2$ inhibited more than half of the inward currents and the deactivating tail currents in wild type- and G199A mutant CIC-2-injected oocytes (Fig. 5A, 1st and 2nd tracings). During cell swelling, $ZnCl_2$ blocked most of the induced time-dependent inactivating outward currents. (Fig. 5A, 3rd and 4th tracings). This is also evident from the normalized current voltage relationships shown for the control and $ZnCl_2$ -inhibited currents recorded in wild

Fig. 4. Hyposmotic cell swelling-stimulated CIC-2 currents.

A. Current traces of a CIC-2 wild type mRNA-injected (upper traces) and a G199A mutant CIC-2 mRNA injected oocyte recorded first in isotonic ND48+96 mM sucrose bath solution (0 min) and then after 15 min (as indicated) of hypotonic swelling in ND48 bath solution (pulse protocol as in Fig. 2). **B.** Current-voltage relationship (arithmetic means $\pm$ SE; $n = 10$) of CIC-2 wild type mRNA-injected oocytes before (open circles) and during hypotonic swelling (15 min; closed triangles). Currents of each oocyte were normalized to the current recorded under control conditions at -140 mV. The maximal currents of the voltage sweeps are depicted. **C.** Current-voltage relationship (arithmetic means $\pm$ SE; $n = 4-9$) in oocytes injected with different mutant CIC-2 mRNAs before (open circles) and after hypotonic swelling (15 min; closed triangles). The amino acid exchange of the mutants is indicated. Currents of each oocyte were obtained as in (B) and normalized to the -140 mV value recorded before swelling. **D.** Conductance change (arithmetic means $\pm$ SE; $n = 4-10$) of wild type and mutant CIC-2 mRNA-injected oocytes evoked by hypotonic swelling (15 min). Conductances before and during hypotonic swelling (15 min) were calculated

by linear regression between 0 and +40 mV and between -60 and -140 mV for the outward and inward currents, respectively. Conductance changes are determined by dividing the conductance of the hypotonically swollen cells by the initial conductance in isotonic bath solution. Data are arithmetic means $\pm$ SE; $n = 4-10$. *, ** and *** indicate significant difference from 1.0 ($p \leq 0.05$, 0.01 and 0.001, two-tailed t-test, respectively).

type (B) and G199A-injected oocytes (C) under isotonic conditions (left) and during cell swelling (right). Taken together, these data strongly suggest, that swelling-stimulated inward currents as well as outward currents were generated by CIC-2.

The swelling-stimulated changes of the inward current were accompanied by alterations of the gating kinetics. $T_{\text{act-swelling-slow}}$ decreased from about 0.5 s (Fig. 3B)

to values below 100 ms (Fig. 6A, B) in wild type and most mutant CIC-2 channels. Following cell swelling, the G199A- and R747H-mutant CIC-2 channels tended to activate slower than wild type CIC-2, an effect reaching statistical significance for (G199A) (Fig. 6B). In addition to faster activation, following cell swelling the current inactivated faster at negative test potentials (see Fig. 4A, upper right). The mean time constant of the inactivation

Fig. 5. ZnCl_2 -sensitivity of the CIC-2-generated currents. *A.* Current traces of a water-injected (upper line), a CIC-2 wild type mRNA-injected (middle line) and a G199A mutant CIC-2 mRNA injected oocyte (lower line) recorded in the absence and presence of ZnCl_2 (1 mM) in isotonic ND48+96 mM sucrose and in hypotonic ND48 bath solution (pulse protocol as in Fig. 2). *B, C.* Current-voltage relationship (arithmetic means $\pm$ SE; $n = 5$ -7) of oocytes injected with wild type (B) or G199A mutant CIC-2 mRNAs (C). Currents were recorded as in (A) in the absence (open circles) and presence (closed triangles) of ZnCl_2 (1 mM) before (left) and after hypotonic swelling (15 min; right). Currents of each oocyte were normalized to the -140 mV value recorded in the absence of ZnCl_2 before swelling.

(T_{deact}) decreased during cell swelling from about 900 ms (Fig. 3D) towards 600 ms in wild type and most mutant CIC-2 channels (Fig. 6C, D). Again, G199A- and the R747H-mutant channels exhibited a significantly slower time constant of deactivation ($T_{\text{deact-swelling}}$) than wild type CIC-2 channels (Fig. 6D). Taken together, the cell swelling experiments suggest an unaltered regulation by cell volume of P48R-, R68H-, and R646Q- mutant channels. R725W-mutant channels which show gating kinetics during cell swelling similar to that of the wild type channel exhibited no swelling-induced activation at hyperpolarizing voltages. Similarly, such swelling-stimulated increase in channel activity at hyperpolarizing voltages was also

absent in G199A- and R747H-mutant channels. In sharp contrast to wild type or R725W channels, cell-swelling did not accelerate the gating kinetics of these two mutants.

Modeling

The general structural homology of two cystathionine β -synthase-domains (CBS) in CIC-channels to a CBS-domain dimer of the bacterial inosine monophosphate dehydrogenase [28] was shown recently ([31] and Fig. 7A). We modeled the first CBS-domain of CIC-2 based on similarity to a dimer-CBS domain from IMPDH. A low-energy structural homology model is shown in Fig. 7B. This model suggests a hydrogen bond between the

Fig. 6. CIC-2 activation and deactivation kinetics in osmotically swollen cells. *A.* Slowly activating currents evoked by a voltage step from -60 mV holding potential to -140 mV voltage recorded first with isotonic medium (ND48+98 mM sucrose) and then upon hypotonic swelling (15 min ND48). Current traces of a CIC-2 wild type mRNA- (left) and a G199A-mutant mRNA-injected oocyte (right) are shown. *B.* Time constant of activation ($T_{\text{act-swelling}}$; arithmetic means $\pm$ SE; $n = 5-10$; determined as in *A* during cell swelling) of wild type and mutant CIC-2 channels (***: $p \leq 0.001$; ANOVA). *C.* Deactivating currents in non-swollen (ND48+98 mM sucrose) and swollen (15min ND48) cells. Currents from a wild type mRNA- (left) and a G199A-mutant CIC-2 mRNA-injected oocyte (right) were de-activated by depolarization from -140 mV test potential to +50 mV voltage. *D.* Time constant of de-activation ($T_{\text{deact-swelling}}$; arithmetic means $\pm$ SE; $n = 5-13$; determined as in *C* during cell swelling) of wild type and mutant CIC-2 channels (*: $p \leq 0.05$, **: $p \leq 0.01$; ANOVA).

backbone of R646 with L614 connecting R646 with the anti-parallel β -sheet of the CBS-domain possibly stabilizing the structure. Recently, the crystal structure of the cytoplasmic domain of the chloride channel CIC-0 has been reported [32]. In this CIC-0 based model Arg646 forms H-bonds with the Pro642 and Arg645 backbones. This may lead to a stabilization of the lower turn terminating the adjacent α -helix (not shown). In both models Arg646 supposedly stabilizes the respective protein conformation. Virtual mutagenesis of R646 to the here reported function-associated mutation Q646 disrupts the hypothetical hydrogen-network.

Discussion

The present study identified variations of the *CLCN2* gene in an African population. These variations comprise modification of the N-terminus (P48R, R68H), of a putative extracellular loop (G199A), and of the C-terminus

(R646Q, R725W, R747H). Regulation of CIC-2 by membrane voltage, cell swelling and pH has been demonstrated to be linked to the cytosolic N-terminus of the channel protein [7, 33]. Fifteen amino acids of the N-terminus have been proposed to form an essential domain that inactivates the channel under resting condition by binding to an internal receptor of the channel protein. Further N-terminal amino acids contribute to this inactivation [7]. More recent data [34], however, argue against such a ball-and-chain mechanism for the regulation of gating by the N-terminal domain (see next paragraph). For CIC-0 a large backbone movement in the C terminus of the channel protein has been functionally linked to slow gating [35].

Time constants of CIC-2 expressed in mammalian cells reportedly differ by up to two orders of magnitude from those of CIC-2 channels expressed in amphibian oocytes. This is possibly due to altered interaction of the CIC-2 channels with Hsp90 in mammalian and amphibian expression systems. Hsp90 has been demonstrated in

filter-forming amino acids. G199A did not affect current or gating kinetics when records were obtained under isosmotic conditions. In contrast to wild type CIC-2, hypotonic swelling, however, did not accelerate the hyperpolarization-stimulated gating of G199A at negative voltage and, thus, did not increase steady state inward current suggesting that 199G contributes to swelling-induced channel activation.

Three further mutations, R646Q, R725W, and R747H were located in the cytosolic C-terminus N-terminal of CBS domain 1, between CBS domain 1 and 2, and C-terminal of CBS domain 2, respectively. CBS domains (named after Cystathionine Beta Synthase [38]) bind adenosyl residues of molecules such as ATP or AMP. The CBS domains of CIC-2 have been demonstrated to similarly bind ATP [39]. Replacement of ATP by AMP reportedly accelerates the opening and closing kinetics of CIC-2 channels [40]. Mutations in this region, such as G715E [20], alter the effect of nucleotides on the channel [40]. Moreover, truncation of CBS-2 probably disrupts CIC channel function as demonstrated for CIC-1 [41]. The mutation R725W of the present study activated faster during hyperpolarization and generated larger steady state currents than the wild type channel when recorded under isosmotic conditions. Similarly, the mutation R747H showed faster activation during hyperpolarization but identical steady state currents as compared to wild type CIC-2. Hypotonic cell swelling did not activate inward currents of R725W or R747H mutant channels. Taken together, these data indicate modulation of the hyperpolarization- or swelling-induced channel gating by mutations in the C-terminus. A third C-terminal mutation R646Q resulted in a strong decrease of the heterologously expressed current as compared to that of the wild type channel. The R646Q-generated current did, however, not differ in gating kinetics or swelling-induced activation from the wild type current suggesting reduced surface expression of R646Q. Modelling of the first CBS-domain of CIC-2 with two independent template structures (CIC-0 [32] and the CBS-domain dimer of the bacterial inosine monophosphate dehydrogenase [28]) suggested a hydrogen bond of R646 with L614 and the Pro642 and Arg645 backbones, respectively. Virtual mutagenesis suggests a disruption of this hydrogen bond in the R646Q mutant which might destabilize the CBS domain. However, we would like to point out that the similarity of CIC-2 with both templates is relatively low. Nevertheless, the data might hint to a role of CBS-1 and R646 in the surface expression of the channel. Even though deletion of CBS-

1 in CIC-1 channels have been demonstrated to be tolerated [41].

The physiological function of the CIC-2 chloride channel in its native system has been discussed intensively [21]. The strong dependence of CIC-2 gating on cell volume and its relatively sensitive volume set-point implicates that CIC-2 might be involved in the regulatory volume decrease (RVD) of eukaryotic cells after cell swelling [7]. However, except in malaria-infected erythrocytes [22], this hypothesis was not supported by experimental data obtained from knock-out models. In oocytes of the nematode *C. elegans*, knockout of CLH-3, the putative orthologue of the human CIC-2, does not affect RVD compared to oocytes from control animals [15]. In this study the authors show that CLH-3 is activated during meiotic maturation and the channel could be identified as a critical component of intercellular communication between oocytes and the surrounding gonadal sheath cells. Transient channel knockdown in this system by RNA-interference affects timing of sheath contractions. The recently established CIC-2 knockout mouse model demonstrates that CIC-2 in mammals is crucially important for retinal and testinal function [21], both cell types functionally dependent on supporting epithelial cells. These findings suggest that CIC-2 has a pivotal function in physiological processes depending on intercellular communication. How far the amino acid exchanges identified in the present study impair those processes await further investigations.

Most of the observed amino acid exchanges are in the intracellular domains of the molecule; also the only coding SNP shared by both chimpanzee and humans is located at this site (at residue 730). Obviously functional constraints are more relaxed in these intracellular domains than in the rest of the molecule that form the pore. Yet, these amino acid exchanges lead to an altered channel function, which may introduce subtle changes in the cell physiology.

Acknowledgements

This study was supported by the 'Schwerpunktprogramm: Life inside cells' of the Deutsche Forschungsgemeinschaft (DFG, La 315/11-1, 11-2 and Hu 781/4-3) and by the Forschungsschwerpunktprogramm des Landes Baden-Württemberg: Dynamik und Modulation zellulärer Infektionsprozesse.

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