**Novel CLCN1 mutations with unique clinical and electrophysiological consequences**

Fen-Fen Wu,1,3 Aisling Ryan,8 Joseph Devaney,3,4 Maike Warnstedt,9 Zeljka Korade-Mirnics,1 Barbara Poser,9 Maria Jose Escriva,9 Elena Pegoraro,1 Audrey S. Yee,5 Kevin J. Felice,6 Michael J. Giuliani,2 Richard F. Mayer,7 Tiziana Mongini,10 Laura Palmucci,10 Michael Marino,4 Reinhardt Rüdel,8 Eric P. Hoffman1,3 and Christoph Fahlke9,11

1 Departments of Human Genetics, Molecular Genetics and Biochemistry and 2 Department of Neurology, University of Pittsburgh, PA, 3 Research Center for Genetic Medicine, Children’s National Medical Center, Washington, DC, 4 Transgenomic Inc., Gaithersburg, MD, 5 Department of Neurology, University of Colorado Health Sciences Center, Denver, CO, 6 Department of Neurology, University of Connecticut Health Center, Farmington, CT, 7 Department of Neurology, University of Maryland Hospital, Baltimore, MD, USA, 8 Department of General Physiology, University of Ulm, Ulm, 9 Institute of Physiology, RWTH Aachen, Aachen, Germany, 10 Department of Neurosciences, University of Turin, Italy and 11 Centro de Estudios Científicos (CECS), Valdivia, Chile

**Summary**

Myotonia is a condition characterized by impaired relaxation of muscle following sudden forceful contraction. We systematically screened all 23 exons of the CLCN1 gene in 88 unrelated patients with myotonia and identified mutations in 14 patients. Six novel mutations were discovered: five were missense (S132C, L283F, T310M, F428S and T550M) found in heterozygous patients, and one was a nonsense mutation (E193X) in a homozygous patient. While five patients had a clinical diagnosis of myotonia congenita, the patient with the F428S mutation exhibited symptoms characteristic of paramyotonia congenita—a condition usually thought to be caused by mutations in the sodium channel gene SCN4A. Nevertheless, no mutations in SCN4A were identified in this patient. The functional consequences of the novel CLCN1 sequence variants were explored by recording chloride currents from human embryonic kidney cells transiently expressing homo- or heterodimeric mutant channels. The five tested mutations caused distinct functional alterations of the homodimeric human muscle chloride ion channel hClC-1. S132C and T550M conferred novel hyperpolarization-induced gating steps, L283F and T310M caused a shift of the activation curve to more positive potentials and F428S reduced the expression level of hClC-1 channels. All showed a dominant-negative effect. For S132C, L283F, T310M and T550M, heterodimeric channels consisting of one wild-type (WT) and one mutant subunit exhibited a shifted activation curve at low intracellular [Cl–]. WT-F428S channels displayed properties similar to WT hClC-1, but expressed at significantly lower levels. The novel mutations exhibit a broad variety of functional defects that, by distinct mechanisms, cause a significant reduction of the resting chloride conductance in muscle of heterozygous patients. Our results provide novel insights into functional alterations and clinical symptoms caused by mutations in CLCN1.

**Keywords:** CLCN1; electrophysiology; mutation; myotonia congenita; voltage-gated chloride channel

**Abbreviations:** CK = creatine kinase; DHPLC = denaturing high-performance liquid chromatography; hClC-1 = human muscle chloride ion channel; SSCP = single-strand conformation polymorphism; WT = wild type

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Introduction

Myotonia congenita is an inherited human muscle disease characterized by muscle stiffness upon sudden forceful movements (Thomsen, 1876; Becker, 1973). The temporarily delayed muscle relaxation is due to hyperexcitability of the skeletal muscle membrane, causing repetitive electrical discharges of affected muscle fibres, the so-called myotonic runs. The clinical features of myotonia congenita include the variable expression of painless muscle stiffness, muscle hypertrophy, and action, percussion and electromyographic myotonia. Myotonic stiffness is typically seen at prolonged rest after exercise and during initiation of movement, but resolves with repetitive muscular contraction (‘warm-up’ phenomenon).

There are two forms of human myotonia congenita, the autosomal dominantly inherited Thomsen’s disease and Becker’s recessive generalized myotonia. These two disease entities differ not only in their inheritance mode, but also in their clinical phenotype. Autosomal recessive myotonia patients usually show an older age of onset (>10 years old) and complain of more severe muscle stiffness and hypertrophy, as well as a peculiar transient muscle weakness in the hands and arms following complete relaxation. With regard to autosomal dominant cases, recent studies have shown variable penetrance and expressivity of the disorder (Koty et al., 1996; Plassart-Schiess et al., 1998), including an unusual case of fluctuating myotonia and weakness occurring only during pregnancies (Lacomis et al., 1999).

The pathophysiological basis of myotonia congenita is well understood. The seminal work of Bryant and colleagues demonstrated a greatly diminished sarcolemmal chloride conductance \( (g_{Cl}) \) in affected muscle fibres (Bryant, 1962) and established that this reduction of \( g_{Cl} \) is the basis for the myotonic phenotype (Bryant and Morales-Aguilera, 1971). Skeletal muscle is unique among excitable tissues in its high resting chloride conductance, which exceeds the resting potassium conductance more than two-fold (Hodgkin and Horowicz, 1959). In the absence of this resting chloride conductance, the length constant of the sarcolemma is significantly increased and therefore elevations of the potassium concentration in the t-tubular lumen during electrical activity cause a depolarization of the sarcolemmal membrane and, consequently, muscle hyperexcitability (Adrian and Bryant, 1974).

In the last few years, distinct allelic mutations in the \( CLCN1 \) gene coding for a muscle-specific chloride channel have been identified in a large number of autosomal dominant (Thomsen) and autosomal recessive (Becker) myotonia cases (Koch et al., 1992; George et al., 1993). To date, 13 missense mutations, an insertion mutation (2512insCTCA) and a nonsense mutation (R894X) have been characterized in Thomsen’s disease patients. Approximately 20 nonsense, splice site and frameshift mutations as well as 25 missense mutations have been reported in Becker’s myotonia patients. The distinct inheritance pattern is due to different functional effects of mutations causing dominant and recessive myotonia. As CIC-type chloride channels are dimeric proteins (Middleton et al., 1994; Fahlke et al., 1997a; Dutzler et al., 2002), heterooligomers consisting of wild-type (WT) and mutant CIC-1 subunits represent a large fraction of the muscle chloride channels in heterozygous patients. The functional properties of these heterooligomeric channels define the inheritance mode of a certain mutation, i.e. mutations exerting a dominant-negative effect by significantly impairing the function of heterodimeric channels cause the dominant trait of myotonia congenita. If heterodimers are functionally normal or only slightly altered, heterozygous patients will have a sufficiently high resting muscle chloride conductance and will not exhibit myotonia.

Myotonia congenita is not the only disease entity with myotonia as a key clinical feature. Other genetic non-dystrophic myotonias such as paramyotonia congenita and potassium-aggravated myotonia (both classical sodium channelopathies; Lehmann-Horn and Jurkat-Rott, 1999) are also associated with myotonia. Although the three diseases can usually be distinguished clinically from each other, sometimes such a differentiation is difficult (Iaizzo et al., 1991; Barchi, 1998; Wagner et al., 1998). We performed a complete screening in all 23 exons of the \( CLCN1 \) gene in 88 unrelated patients exhibiting myotonia as a symptom. In addition to six previously reported mutations (G285E, I290M, R338Q, F413C, R894X and donor splice site 1471 +1 G→A), four novel missense mutations (S132C, L283F, T310M and T550M) and one nonsense mutation (E193X) were identified in a total of 13 patients with myotonia congenita. In one patient classified as a case of paramyotonia congenita (Weiss and Mayer, 1997), another novel \( CLCN1 \) missense mutation (F428S) was found. We characterized the electrophysiological consequences of the novel missense mutations, and correlated the molecular findings with the clinical phenotype of the corresponding patients.

Material and methods

Patient selection

The 88 subjects included in this study were selected from patient samples referred to Dr Hoffman’s laboratory for molecular diagnostic testing at the Research Center for Genetic Medicine, Children’s National Medical Center (previously at the University of Pittsburgh). All studies were approved by the institutional board of the University of Pittsburgh and Children’s National Center and included a subject consent form entitled ‘Candidate gene studies in neurological disorders’. Signed consent forms permitted the inclusion of subjects in this study. Subjects were not examined at a centralized location, and the inclusion criteria were relaxed relative to previous studies; only electrical and/or symptomatic evidence of myotonia by physician report was required for inclusion in the study. This relaxed inclusion criterion meant that a broader range of symptoms was studied; however, this also meant that a smaller proportion of patients...
Table 1 *Main clinical findings in the patients investigated*

<table>
<thead>
<tr>
<th>Patient</th>
<th>Mutation</th>
<th>Age of onset (years)</th>
<th>Frequency of attacks</th>
<th>Weakness</th>
<th>Warm up</th>
<th>Additional symptoms</th>
<th>EMG</th>
<th>CK (U/l)</th>
<th>Muscle biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S132C</td>
<td>Adolescence</td>
<td>Variable through adolescence, then again in late 20s, then constant leg cramping and stiffness</td>
<td>Muscle spasms in hands during repetitive manual tasks</td>
<td>Yes</td>
<td>Bulky muscles without any weight lifting</td>
<td>Diffuse myotonic discharges in arms, hands, legs and feet</td>
<td>Not performed</td>
<td>Not performed</td>
</tr>
<tr>
<td>2</td>
<td>E193X</td>
<td>25</td>
<td>Constant muscle stiffness with mild daytime variability</td>
<td>Occasional hand weakness</td>
<td>Yes</td>
<td>Diffuse and abundant myotonic discharges in all muscles examined</td>
<td>107</td>
<td>Nonspecific changes with increased variation in fibre size and &gt;10% internal nuclei</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>L283F</td>
<td>7</td>
<td>Constant muscle stiffness, one episode of severe stiffness</td>
<td>None</td>
<td>Yes</td>
<td>Diffuse and abundant myotonic discharges in all muscles examined</td>
<td>304</td>
<td>Not performed</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>T310M</td>
<td>31</td>
<td>During pregnancy</td>
<td>During pregnancy</td>
<td>No</td>
<td>Grip myotonia</td>
<td>Diffuse myotonia</td>
<td>&lt;20</td>
<td>Not performed</td>
</tr>
<tr>
<td>5</td>
<td>F428S</td>
<td>40</td>
<td>Constant muscle stiffness increased by cold</td>
<td>Hand weakness after strong exercise or cooling</td>
<td>Mild, but strong exercise results in weakness and myotonia on EMG</td>
<td>Hand paraesthesias</td>
<td>Bursts of repetitive discharges and myotonia enhanced by cooling</td>
<td>100</td>
<td>Not performed</td>
</tr>
<tr>
<td>6</td>
<td>T550M</td>
<td>7</td>
<td>Constant muscle stiffness with occasional fluctuations</td>
<td>Mild proximal weakness</td>
<td>Yes</td>
<td>Myotonic discharges, occasional polyphasia</td>
<td>225</td>
<td>Fibre size variability, angular fibres, central nuclei</td>
<td></td>
</tr>
</tbody>
</table>
tested mutation positive for chloride channel gene changes. Below, the clinical features of patients that tested positive for sequence changes in the CLCN1 gene are summarized. Additionally, the main distinguishing features of each of the patients are shown in Table 1.

Patient 1: myotonia congenita, CLCN1-S132C mutation
A 40-year-old man, GR, complained of muscle pain and cramp in his back and legs. The patient reported that his symptoms began at puberty and he suffered stiffness and spasms on occasion, especially during physical education classes. He experienced muscle ‘lock-up and freeze’ when he tried to run quickly on the track and field. In his late 20s, he developed an exacerbation of symptoms with more cramping. Following a symptom-free period in his early-to-mid 30s, his condition once again deteriorated in his late 30s. He had prominent muscle hypertrophy, most notably in biceps, deltoids, thenar and hypothenar eminences and gastrocnemii. He denied any difficulty with speech, swallowing or sphincter control. There was no prior history of muscle disease in the family, however his 16-year-old son complained of muscle stiffness with onset at puberty. The patient had significant percussion myotonia of both thenar eminences and bilateral grip myotonia, but no facial weakness or lid myotonia. An EMG study showed myotonic discharges in multiple muscles tested.

Patient 2: myotonia congenita, CLCN1-E193X mutation
A 44-year-old man, AG, complained of muscle stiffness and cramps, especially during the initial minutes of performing an activity (e.g. using his hands or walking). He first noticed the symptoms in his early 20s. None of his nine siblings or parents had a similar problem. Two of his three children, however, complained of leg pain and difficulty with running in their first decade. Clinical examination was remarkable for diffuse and severe generalized action, and percussion myotonia affecting eyelid, thigh, hand and arm muscles. At least 10 s were required for the action myotonia to abate, even after brief minor contractions in the muscles required for hand grip, eyelid closure and walking. The muscle stiffness improved mildly with repetitive activity and was not worsened by cold exposure. In general, muscle strength appeared to be normal, although there was definite transient weakness during periods of more intense myotonia. The patient had prominent muscle bulk with a short stature, but no dysmorphic features. DNA studies for myotonic dystrophy were negative for the trinucleotide repeat expansion. The serum creatine kinase (CK) values and ECGs were normal. Muscle biopsy slides (paraffin sections) showed non-specific changes, with increased variation in fibre size and >10% of internalized nuclei. No necrosis, vacuoles, inflammation, regeneration or dystrophic changes were noted.

Patient 3: myotonia congenita, CLCN1-L283F mutation
The proband, SM, was a 22-year-old male with clinical myotonia since childhood. During his first school years, one episode of acute stiffness of the lower limbs occurred in the morning, with recovery within a few hours. Neurological examination showed diffuse muscle hypertrophy and myotonia with warm-up phenomenon. His muscle strength was normal and tendon reflexes were reduced. Serum CK was slightly elevated at 304 U/l (normal <200 U/l). ECG was normal. No other family members had similar complaints.

Patient 4: myotonia congenita, CLCN1-T310M mutation
A 31-year-old woman, LK, first observed that she was unable to lift heavy objects in her late teens. During the first trimester of her first pregnancy she complained of weakness and noted that she was unable to readily release a gripped object from her right hand. She had difficulty turning her head to look over her shoulder while she was driving. She also noticed that her lower extremities were stiff and heavy when she began walking after a period of rest. She had no complaints of muscle cramps or pain. Her symptoms did not improve with time, temperature, or level of activity. There was no family history of a similar condition, nor complaint of muscle weakness during pregnancy. Neurological examination revealed mild muscle weakness of neck flexors and extensors, as well as proximal more than distal involvement of the upper and lower extremities. EMG examinations demonstrated electrical myotonia in most muscles examined during pregnancy, but the myotonia showed a significant decrease after delivery. Cold immersion test for paramyotonia and short and prolonged exercise testing were unremarkable.

Patient 5: paramyotonia congenita, CLCN1-F428S mutation
This patient’s clinical data have been published without mutation data (Weiss and Mayer, 1997). This 47-year-old woman experienced muscle stiffness, mild weakness and pain, with symptoms worsening upon cooling and exercise. Stiffness was reduced by mild exercise. Strong exercise reduced the stiffness further, but resulted in weakness and increased myotonia on EMG. The EMG features of this patient showed prominent bursts of repetitive discharges at room temperature, but decreased repetitive discharges upon cooling of the muscle, while the myotonic discharges became prominent only upon cooling. Following exercise, the myotonic discharges increased.
Patient 6: myotonia congenita CLCN1-T550M mutation

The proband, PRM, was a 62-year-old female who complained of difficulty climbing stairs and sudden stiffness upon rapid movement since childhood. In her late 30s to early 40s, she experienced episodes of generalized stiffness leading to occasional falls and injuries. She was first examined in 1983 because of muscle weakness and myalgia. Neurological examination revealed myotonia in both hands without clear muscle weakness. Serum CK was mildly elevated (225 U/l). An EMG showed myotonic discharges and occasional short polyphasic potentials. ECG showed incomplete right bundle block. Muscle biopsy showed muscle fibre variability with several small angular fibres and many central nuclei, often in longitudinal chains. In the following years the patient developed moderate proximal muscle weakness and dysphagia. Videofluorography demonstrated swallowing dyskinesia and slow transit.

The proband’s son, PG, was 24 years old and complained of occasional muscle stiffness. Neurological examination was unremarkable apart from slight calf hypertrophy. He had no clinical myotonia but showed some EMG myotonic discharges in his hands. The proband’s nephews, LM and LO, aged 38 and 34 years, respectively, complained of occasional muscle stiffness. Neurological examination revealed myotonia in both hands without clear muscle weakness. The proband’s nephews, LM and LO, aged 38 and 34 years, respectively, complained of occasional stiffness but were not examined.

Molecular genetic analysis

Genomic DNA was extracted from 10 ml of whole blood collected in a blood collection tube (EDTA) as described previously (Miller et al., 1988). Patient DNA samples were first screened for the most common dominant CLCN1 mutation, G230E, by amplification-refractory mutation system analysis (Newton et al., 1989). The entire coding sequence of the CLCN1 gene (23 exons) was screened for mutations using either single-strand conformation polymorphism (SSCP) [4°C and mutation detection enhancement (MDE) gels; FMC Corp, Westchester, PA, USA] or denaturing high-performance liquid chromatography (DHPLC), followed by DNA sequencing as described previously (Hoffbuhr et al., 2001; Wu et al., 2001). We have found sensitivity of DHPLC to be nearly 100% (Hoffbuhr et al., 2001); however, SSCP is not as sensitive (∼70% in our hands).

Additionally, in patients who tested positive for CLCN1 mutations, the whole coding sequence of SCN4A was screened using similar techniques to exclude mutations in the skeletal muscle sodium channel (Wu et al., 2001).

Expression of hClC-1 channel constructs

Mutant channels were expressed in human embryonic kidney (HEK)-293 and tsA201 cells by transfecting 2 μg of plasmid DNA into 10-mm plates using a calcium phosphate precipitation method as described previously (Fahlke et al., 1997b). To identify cells with a high probability of expressing recombinant ion channels, cells were cotransfected with a plasmid encoding the CD8 antigen and incubated 5 min before use with polystyrene microbeads precoated with anti-CD8 antibody (Dynabeads M-450 CD 8; Dynal, Great Neck, NY, USA) (Jurman et al., 1994). Only cells decorated with microbeads were used for electrophysiological recordings. In some cases, cells were transiently transfected with the construct and a pEGFP-N1 reporter plasmid using Effectene™ Transfection Kit (Qiagen, Hilden, Germany). Cells were typically examined 2 days after transient transfection. We did not observe any differences between results obtained in tsA201 and HEK-293 cells or with the different transfection methods. For some experiments examining WT hClC-1, stably transfected HEK-293 cells (Fahlke et al., 1996) were used. Functional properties of stably expressed channels and those transiently expressed were indistinguishable.

Electrophysiology

Standard whole-cell or excised outside-out patch-clamp recordings were performed using an Axopatch 200B (Axon Instruments, Union City, CA, USA), an EPC8 or an EPC9 (HEKA Elektronik, Lambrecht, Germany) amplifier. Pipettes were pulled from borosilicate glass and had resistances of 0.8–2.2 MΩ. For excised patches, pipettes were coated with wax for reduction of pipette capacitance. More than 80% of the series resistance was compensated by an analogue procedure. The calculated voltage error due to series resistance was always <5 mV. Currents were filtered with an internal 4-pole Bessel filter with 1 kHz (~3 dB) and digitized with sampling rates of 5 kHz using a Digidata AD/DA converter (Axon Instruments, Union City, CA, USA). Contribution of leak current was negligible under our conditions, and no correction for leakage current was used. Two distinct pipette (intracellular) solutions were used:
standard intracellular solution containing 120 mM NaCl, 2 mM MgCl₂, 5 mM EGTA, 10 mM HEPES, pH 7.4; and low intracellular chloride solution containing 120 mM Na-glutamate, 2 mM MgCl₂, 5 mM EGTA and 10 mM HEPES, pH 7.4. To connect the low [Cl⁻] solution to the amplifier, agar bridges containing 3 M KCl in 0.03% agar were used. The extracellular solution contained: 140 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH 7.4. Junction potentials calculated using the JPCalc software (Barry, 1994) and offset potentials measured at the end of each experiment were used to correct results. Cells were held to potentials close to the calculated chloride reversal potential to prevent shifts of the intracellular [Cl⁻] during prolonged recordings, i.e. to 0 mV (for standard intracellular solution) or ±100 mV (for low intracellular [Cl⁻] solution) for at least 5 s between two test sweeps. To obtain the voltage dependence of activation, the instantaneous current amplitude determined 200 μs after a voltage step to −125 mV was measured after prepulses to different voltages and then divided by its maximum value. The normalized data were then plotted versus the preceding potential. This plot yields the voltage dependence of the relative open probability, P_open, at the end of the prepulse. The steady-state activation curve was obtained using 0.4-s prepulses and fitted with a single Boltzmann term and a voltage-independent value: I(V) = Amp/[1 + e^(-V - V_0.5/kV)] + constant. All data are shown as means ± standard error of the mean from at least four cells. For statistical evaluation, the Student's t-test was applied with P < 0.05 indicating significance.

Results
Identification of CLCN1 mutations
The selected 88 unrelated myotonia patients were tested for sequence changes in all 23 exons of the CLCN1 gene, using either SSCP methods (30 patients) or DHPLC methods (58 patients). We identified six previously reported mutations (G285E, I290M, R338Q, F413C, R894X and donor splice site 1471 +1 G) in eight patients and six novel mutations in six patients. The six novel mutations showed unique conformers by SSCP in the MDE and the 4°C gels in exons 7, 8, 12 and 15 (Fig. 1).

DNA sequencing showed each of these patients to be heterozygous for novel missense mutations (Fig. 1). The four novel mutations were: (i) C-to-T transition at coding sequence 847 (847C>T), which resulted in the substitution of phenylalanine for leucine at amino acid 283 position (L283F) in exon 7; (ii) C-to-T transition at coding sequence 929 (929C>T), which resulted in the substitution of methionine for threonine at amino acid 310 position (T310M) in exon 8; (iii) T-to-C transition at coding sequence 1283 (1283T>C), which resulted in the substitution of serine for phenylalanine at amino acid 428 position (F428S) in exon 12; and (iv) C-to-T transition at coding sequence 1649 (1649C>T), which resulted in the substitution of methionine for threonine at amino acid 550 position (T550M) in exon 15. Mutations were first studied in immediate family members and in normal populations to exclude possible polymorphic variants. The T310M substitution created an NlaIII restriction enzyme site in the amplicon of exon 8. As family members were unavailable for testing, 50 control DNA samples (100 chromosomes) were tested and none showed the T310M change (data not shown). The T550M substitution created an Asel endonuclease restriction enzyme site in the amplicon of exon 15. The mutation was also present in an affected brother and in both the patient’s son and her brother’s son, indicating dominant transmission (data not shown). Analysis of 50 control DNA samples did not identify this change (data not shown). As neither L283F nor F428S substitutions change a restriction enzyme site, 50 control samples were evaluated by DNA sequencing. None showed these sequence alterations (data not shown).

Two of the six novel mutations were found by DHPLC heteroduplexes in exons 3 and 5 (Fig. 2). The two novel mutations were: (i) A-to-T transversion at coding sequence 394 (394A>T), which resulted in the substitution of cysteine for serine at amino acid 132 position (S132C) in exon 3; and (ii) G-to-T transversion at coding sequence 577 (577G>T), which resulted in the substitution of a stop codon for glutamate at amino acid 193 position (E193X) in exon 5, leading to a truncation of the chloride channel protein. The S132C substitution was present in an affected son, indicating dominant transmission (data not shown). Analysis of 65 control DNA samples did not identify such a change (data not shown). There were no family members available to test the transmission pattern of the E193X mutation, therefore 65 control DNA samples (130 chromosomes) were analysed by DHPLC and did not show heteroduplexes (data not shown).

Patient 4 carried a diagnosis of paramyotonia congenita, a disease generally caused by mutations in a voltage-gated sodium channel (Ptacek et al., 1992). In a complete screen of the entire coding region of SCN4A, we did not find any mutations in the sodium channel gene.

DHPLC is considered highly sensitive for detection of sequence changes, and we have found 100% sensitivity in previous studies (Hoffbuhr et al., 2001). However, in the 30 patients studied by SSCP alone, sensitivity is not as good, and it is possible that some CLCN1 sequence alterations were missed.

Functional characterization of WT and mutant channels
The five novel missense mutations (S132C, L283F, T310M, F428S and T550M) were introduced into a full-length human muscle chloride channel, heterologously expressed in mammalian cells and investigated using the patch-clamp technique. The E193X mutation causes the deletion of ~80% of the coding sequence of hClC-1 containing major determinants of the ion conduction pathway (Fahlke et al., 1997c;
Dutzler et al., 2002) and is thus expected to be unable to form a functional channel. We therefore decided not to study this mutation electrophysiologically.

The skeletal muscle chloride channel is a dimeric protein (Fahlke et al., 1997a), and in heterozygous patients a significant fraction of ClC-1 channels are expected to be...
heterodimeric channels consisting of one WT and one mutant subunit. All patients with mutations that were examined electrophysiologically are heterozygous, and thus the properties of heterodimeric channels are of major importance in understanding the pathophysiological consequences of these mutations. To study the functional properties of heterodimeric channels, we expressed concatameric constructs that link one WT and one mutant sequence in a single open reading frame in tsA201 cells. This allows the study of a homogenous population of heterodimeric channels by whole-cell or excised outside-out patch-clamp recordings (Fahlke et al., 1997).

All constructs were tested using two internal solutions: a standard solution containing 124 mM Cl⁻ and a low [Cl⁻] internal solution ([Cl⁻] = 4 mM). The first condition has been used in the majority of reports about WT and mutant hClC-1 so far. In muscle fibres, however, the internal Cl⁻ concentration is very low (Dulhunty, 1978). As permeant anions are known to affect the gating of CIC channels (Richard and Miller, 1990; Fahlke et al., 1995; Pusch et al., 1995), we felt it necessary to study mutant channels under a physiological internal Cl⁻ concentration.

**Electrophysiological analysis of WT hClC-1 channels**

Figure 4 shows representative current recordings from cells expressing WT hClC-1 channels. Upon voltage steps to hyperpolarizing potentials, chloride current amplitudes rose instantaneously and then deactivated on a slower time scale to a non-zero steady-state level (Fig. 4A and B). Current responses to membrane depolarization from a holding potential of 0 mV were time-independent (Fig. 4A), while from a holding potential of −100 mV an instantaneous rise was followed by a slower, biexponential rise due to channel activation (Fig. 4B). The instantaneous current amplitudes were strongly inwardly rectifying for high as well as for low [Cl⁻] internal conditions (Fig. 4C and D). The relative open probability of WT hClC-1 shows a minimum non-zero value at potentials negative to −100 mV and increases with more depolarized potentials (Fig. 4E and F). At the normal resting potential of muscle (−85 mV), a significant percentage of WT hClC-1 channels is open under both ionic conditions. The voltage dependence of activation differs for the two tested ionic conditions; at low [Cl⁻] the activation curve is shifted to more hyperpolarized potentials (Table 2). This shift increases the percentage of channels open under resting conditions, i.e. the relative open probability is 0.25 ± 0.06 (n = 6) for symmetrical chloride, and 0.49 ± 0.05 (n = 5) for low intracellular [Cl⁻].

**Functional properties of homodimeric mutant channels**

All tested mutant channels were functional and expressed well with whole-cell peak current amplitudes at −125 mV.
between 2 and 20 nA. Figure 5 shows representative current recordings from cells transfected with the five mutant pReCMV-hClC-1 plasmids. Inspection of representative current recordings showed clear functional alterations for four of the five novel myotonia mutations. Using standard solutions, S132C activates upon membrane hyperpolarization, in clear contrast to the depolarization-induced activation of WT hClC-1 channels (Fig. 5A). This hyperpolarization-induced activation requires a high internal [Cl\(^-\)], as at a low internal [Cl\(^-\)] current responses to the same voltage steps are almost time independent (Fig. 5B). At physiological internal [Cl\(^-\)], S132C mutant channels exhibited only a minimum open probability over the whole voltage range and this caused a drastic decrease of macroscopic currents in cells expressing mutant channels (Fig. 5C) compared with cells expressing WT hClC-1.

L283F hClC-1 channels displayed deactivation gating upon hyperpolarization as well as activation upon depolarization for symmetrical (Fig. 5D) and for low internal [Cl\(^-\)]int (Fig. 5E). Nevertheless, the voltage dependence of mutant channels was shifted to more positive potentials as compared with WT channels (Fig. 5F; Table 2). This alteration of ClC-1 gating caused a significant reduction of the relative open probability at –85 mV, to 0.20 ± 0.05 (n = 4) for [Cl\(^-\)]int = 124 mM, and 0.01 ± 0.01 (n = 4) at [Cl\(^-\)]int = 4 mM, respectively. As the resting chloride conductance is proportional to this open probability, these values predict a significant reduction of gCl in patients’ muscle.

T310M channels exhibited a unique dependence of channel gating on the intracellular [Cl\(^-\)]. While mutant channels appeared quite similar to WT channels under standard solutions (Fig. 5G), the voltage dependence of activation is pronouncedly shifted to more depolarized potentials at low internal chloride (Fig. 5H and I; Table 2). This shift caused a strong reduction of the open probability at –85 mV, producing a decreased macroscopic chloride conductance.

The functional properties of F428S hClC-1 channels were indistinguishable from those of WT channels at both chloride concentrations (Fig. 5J and K). The activation curve of F428S channels was also similar to that of WT channels (Table 2). The major difference between F428S and WT was a reduced current density. WT channels expressed very robustly with peak amplitudes often >10 nA at –125 mV. In cells expressing F428S, current amplitudes were significantly lower (instantaneous current amplitude at –125 mV for [Cl\(^-\)]int = 4 mM; WT: ±6.9 nA, n = 6; F428S: ±1.5 ± 0.2 nA, n = 6; P < 0.01) (Fig. 5L).

T550M homodimeric channels exhibited an unprecedented alteration of hClC-1 gating. Upon hyperpolarizing voltage steps, mutant channels first display a fast deactivation and then a consecutive slower activation (Fig. 5M and N). The existence of two gating processes with inverse voltage dependence produced an inverse bell-shaped activation curve at high internal [Cl\(^-\)]int, causing the relative open probability of mutant channels at –85 mV to be higher than that of WT. In cells perfused with low chloride internal solution, the hyperpolarization-induced activation was less pronounced and therefore mutant channels exhibited a lower relative open probability than WT under these ionic conditions (Fig. 5O).

### Functional properties of heterodimeric mutant channels

Heterodimeric channels were all functional and expressed at levels similar to the corresponding homodimeric channels. Whole-cell peak current amplitudes at –125 mV were between 2 and 20 nA for each of the five mutant channels. Surprisingly, despite the differing functional properties of homodimeric mutant channels, four of the five studied heterodimeric channels showed very similar changes, suggesting a shared dominant-negative effect (Fig. 6).

### Table 2: Boltzmann parameters of the activation curves of different ClC-1 constructs. Empty fields are given, if the construct is not existing (heterodimeric WT channels) or if the activation curve cannot be fitted with a single Boltzmann function

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Homodimeric channels [Cl(^-)]int = 124 mM</th>
<th>Homodimeric channels [Cl(^-)]int = 4 mM</th>
<th>Heterodimeric channels [Cl(^-)]int = 124 mM</th>
<th>Heterodimeric channels [Cl(^-)]int = 4 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>v0.5 = –49.9 ± 0.5 mV, kV = 22.7 ± 0.4 mV</td>
<td>v0.5 = –85.7 ± 3.9 mV, kV = 36.6 ± 3.8 mV</td>
<td>v0.5 = –64.6 ± 16.5 mV, kV = 34.7 ± 12.7 mV</td>
<td>--</td>
</tr>
<tr>
<td>S132C</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>v0.5 = 58.4 ± 8.6 mV, kV = 39.6 ± 1.3 mV</td>
</tr>
<tr>
<td>L283F</td>
<td>v0.5 = 64.6 ± 16.5 mV, kV = 34.7 ± 12.7 mV</td>
<td>v0.5 = 133.3 ± 23.8 mV, kV = 66.8 ± 7.7 mV</td>
<td>v0.5 = 34.6 ± 11.6 mV, kV = 53.1 ± 7.2 mV</td>
<td>v0.5 = 34.6 ± 11.6 mV, kV = 53.1 ± 7.2 mV</td>
</tr>
<tr>
<td>T310M</td>
<td>v0.5 = –55.5 ± 3.8 mV, kV = 35.0 ± 1.4 mV</td>
<td>v0.5 = 110.8 ± 13.8 mV, kV = 76.3 ± 11.4 mV</td>
<td>v0.5 = –61.9 ± 2.7 mV, kV = 29.5 ± 2.5 mV</td>
<td>v0.5 = –75.7 ± 2.9 mV, kV = 39.4 ± 2.8 mV</td>
</tr>
<tr>
<td>F428S</td>
<td>v0.5 = –49.4 ± 1.5 mV, kV = 20.7 ± 1.4 mV</td>
<td>v0.5 = –87.3 ± 2.6 mV, kV = 33.3 ± 4.8 mV</td>
<td>v0.5 = –47.6 ± 2.5 mV, kV = 20.7 ± 1.5 mV</td>
<td>v0.5 = –94.6 ± 7.0 mV, kV = 25.1 ± 0.7 mV</td>
</tr>
<tr>
<td>T550M</td>
<td>--</td>
<td>v0.5 = –8.0 ± 3.3 mV, kV = 61.6 ± 4.1 mV</td>
<td>v0.5 = –79.4 ± 0.8 mV, kV = 21.5 ± 0.7 mV</td>
<td>v0.5 = 58.0 ± 9.3 mV</td>
</tr>
</tbody>
</table>
WT-S132C heterodimeric channels deactivated upon hyperpolarization at both tested internal solutions. At high internal chloride, deactivation was less complete than for WT channels (Fig. 6A), due to an increased minimum open probability at very negative potentials (Fig. 6C). The voltage dependence of activation could not be fitted with a single Boltzmann function, but instead required an additional term with a midpoint at very positive potentials (Fig. 6C). Under physiological \([\mathrm{Cl}^-]_{\text{int}}\), the activation curve of WT-S132C was shifted dramatically to the right (Table 2). This reduced the relative open probability at the muscle resting potential to 0.05 ± 0.01 (n = 5). This loss of function of the heterodimeric channels is consistent with a dominantly inherited myotonia. WT-L283F and WT-T310M heterodimers are functionally similar to WT-S132C channels (Fig. 6D, E, G and H). For all three mutations, deactivation is less complete at high internal chloride, and the activation curve is shifted to more positive potentials for low internal chloride (Fig. 6F and I; Table 2).

T550M showed a dominant-negative effect, which was only apparent at a low internal \([\mathrm{Cl}^-]_{\text{int}}\). At high internal chloride, the activation was basically unshifted compared with WT. Lowering the internal chloride results in a shift to more positive potential and a less steep voltage dependence. These two changes caused a reduction of the relative open probability to 0.38 ± 0.03 under a physiological internal \([\mathrm{Cl}^-]\) at −85 mV.

The dominant-negative effect of F428S was qualitatively different from the other mutations. Similar to F428S homodimeric channels, WT-F428S heterodimeric channels exhibited gating properties that were indistinguishable from WT channels. Nevertheless, the absolute current amplitude measured in cells expressing the WT-F428S concatameric construct was significantly lower than in cells expressing WT channels (instantaneous current amplitude at −125 mV for \([\mathrm{Cl}^-]_{\text{int}} = 4 \text{ mM}\); WT: −6.9 ± 1.7 nA, n = 7; WT-F428S: −0.5 ± 0.1 nA, n = 4; P < 0.01).

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**Discussion**

In this study, we conducted a systematic mutation screening in all 23 exons of the *CLCN1* gene in 88 unrelated familial and sporadic myotonia patients through SSCP, DHPLC and sequencing methods. We have identified six previously reported mutations (G285E, I290M, R338Q, F413C, R894X and donor splice site 1471 +1 G®A), and six novel mutations (S132C, E193X, L283F, T310M, F428S and T550M) (Fig. 3) in 14 unrelated subjects.

We have characterized the molecular genetic and functional consequences of the novel mutations through a variety of approaches. First, none of the six novel mutations was present in >50 control DNA samples (100 chromosomes), arguing against these changes being polymorphisms. Secondly, we tested for conservation of the amino acid substitutions in the ClC-1 proteins across different species (Figs 1 and 2). Each missense mutation caused rather dramatic amino acid changes in conserved residues and were thus predicted to modulate channel function. Thirdly, and most importantly, changes of chloride channel function were documented for each of the five missense mutations by electrophysiological studies.

All the tested patients with missense mutations were heterozygous and therefore have one mutant and one normal *CLCN1* gene copy. Thus, if the mutant expresses at levels similar to WT, three classes of dimeric muscle chloride channels exist in the sarcolemma of these patients, with a relative proportion of 25% WT homodimers, 50% heterodimers and 25% mutant homodimers. A critical issue to address in the electrophysiological studies is whether heterodimeric and mutant homodimeric channels show different or similar electrophysiological abnormalities. We examined each mutant channel as homodimer and heterodimer, and could in fact demonstrate that these distinct channel populations show different functional abnormalities. This means that multiple types of electrophysiological defects co-exist in patient muscle and, furthermore, that the relative ratios of the different classes of channel dimers could be a major determinant of severity and subtype of myotonia. It is conceivable that disease-causing mutations affect the ability of mutant subunits to form homo- or heterodimeric channels. In heterozygous patients expressing such a mutant CIC-1 protein, the distributions of channels will deviate from the 25% : 50% : 25% ratio given above. To test for such effects in a heterologous expression system is difficult and we did not address such a possibility in our studies.

A second variable tested was the effect of different intracellular chloride concentrations on channel function. While the great majority of *CLCN1* functional studies have only been done at a non-physiological, high internal chloride, we also studied channel function at a physiological, low internal \([\mathrm{Cl}^-]\). We often found dramatically different abnormalities between the two intracellular \([\mathrm{Cl}^-]\), as described.
below. Thus, intracellular \([\text{Cl}^-]\) in patient muscle would be expected to have an effect on channel function and thus on resulting patient symptoms. In control muscle, the high resting chloride conductance clamps \([\text{Cl}^-]\) to low values. In contrast, myotonic muscle exhibiting a greatly reduced \(g_{\text{Cl}}\) intracellular \([\text{Cl}^-]\) might undergo certain variations due to regulated ion transport processes, and this dependence could therefore contribute to the variability of clinical symptoms with time that is sometimes observed. As the sarcolemmal chloride conductance itself is an important parameter defining this value, this dependence further increases the complexity of functional effects of \(\text{CLCN1}\) mutations.

The tested patients exhibited a considerable variability of symptoms. One had the typical symptoms of paramyotonia congenita, and another patient was without any symptoms before and after pregnancy. Within the remaining cases of classical myotonia congenita, there is nevertheless a correlation between the severity of symptoms and inheritance pattern and the functional effects of the studied mutations. The recessive patient exhibiting the E193X mutation suffers the most severe symptoms. He is homozygous, and there is little doubt that a deletion of \(-80\%\) of the coding region will cause a non-functional channel protein, and thus a complete absence of resting chloride conductance. For S132C, L283F and T550M, homodimeric mutant and heterodimeric WT-mutant channels exhibit functional alterations causing a reduced open probability, and thus a reduced conductance at resting potential. Assuming equal expression and free assembly of WT and mutant subunits in heterozygous muscle, one can make a prediction of the relative resting conductance in the sarcolemma of heterozygous patients, and this value decreases in the order of T550M, L283F and S132C, in accordance with the severity of clinical symptoms.

The S132C mutation co-segregated with the phenotype in an autosomal dominant fashion, with both the affected father and son complaining of similar symptoms and carrying the same mutation. Both the father and son showed unusually late onset (after 11 years of age) and unusually severe myotonia. S132C homodimeric channels activated upon hyperpolarization (Fig. 5A) and this activation process depended on the internal \([\text{Cl}^-]\), in clear contrast to activation of WT channels (Fig. 4A). At a physiological low \([\text{Cl}^-]\) to low values, the relative open probability of S132C hClC-1 was very small and voltage-independent. Previously, two different mutations have been...
reported that result in a hyperpolarization-induced activation and a coupling of channel opening to the internal \([\text{Cl}^-]\) (D136G and G499R) (Fahlke et al., 1995; Zhang et al., 2000a). Both were found in recessive myotonia, and S132C is thus the first mutation found in a patient with Thomsen’s myotonia that causes this particular alteration of hClC-1 gating. The dominant S132C mutation and the recessive D136G mutation are in the same region of the channel, and both homodimeric channels show quite similar functional changes. Heterodimeric channels, however, exhibit distinct functional changes. WT-D136G heterodimeric channels display a less complete activation at negative potentials resulting in an increased resting open probability (Fahlke et al., 1997a). This feature accounts for a normal chloride ion conductance in heterozygous patients and explains the recessive inheritance mode of the D136G mutation. In contrast, WT-S132C heterodimers exhibit a rightward shift of voltage dependence of activation at low physiological internal \([\text{Cl}^-]\) (Table 2). This reduces the relative open probability at \(-85\) mV to a very low value and fully explains the dominant inheritance of this mutation. Surprisingly, two mutations causing a similar alteration of gating (i.e. an inversion of the voltage dependence of activation) and located in the same channel region, interact in a discriminative fashion with a WT protein in a heterodimeric channel.

The patients carrying the L283F mutation showed an early onset of myotonia, with diffuse muscle hypertrophy and a warm-up phenomenon. L283F homodimeric channels exhibited a depolarization-induced activation with a voltage dependence that was significantly shifted to more depolarized potentials at a high and at a low internal \([\text{Cl}^-]\). In contrast, heterodimeric channels show only slight alterations of gating at high \([\text{Cl}^-]\) but a pronounced shift of the activation curve.
at a physiological $[\text{Cl}^-]_{\text{int}}$. Under these conditions, the open probability of WT-L283F at $-85$ mV is greatly reduced compared with WT. Thus, the electrophysiological results predict a significantly reduced sarcolemmal chloride conductance in heterozygous patients.

The electrophysiological characterization of the T310M mutation at a physiological low internal $[\text{Cl}^-]$ revealed significant functional alterations of homo- and heterodimeric T310M channels that predict a drastic reduction of the sarcolemmal $g_{\text{Cl}}$. This was a rather surprising finding as myotonia and weakness first manifested during pregnancy and decreased after delivery. The clinical symptoms, together with the functional properties of mutant channels in a heterologous expression system, indicate that the hormonal status somehow influenced chloride channel function. An intriguing possibility might be that the intracellular $[\text{Cl}^-]$ is under the control of hormone-regulated transport mechanisms (Lacomis et al., 1999). While outside pregnancy $[\text{Cl}^-]_{\text{int}}$ would be sufficiently high to allow an almost normal function of mutant channels (Table 2), a reduction during pregnancy could cause a pronounced dysfunction and thus myotonia. It is also conceivable that the expression levels of the mutant and the WT protein are somehow regulated by hormones. Unfortunately, the specific molecular or physiological interactions between hormones and ionic concentrations or ion channel function are currently not sufficiently understood and are beyond the scope of this article. Pregnancy-induced myotonia and weakness have been described before for patients having the more common dominant G230E mutation (Lacomis et al., 1999).

The patient with the T550M mutation experienced muscle stiffness upon rapid movement since early childhood and episodes of generalized stiffness in her late 30s and 40s. Homodimeric T550M channels exhibit a depolarization- as well as a hyperpolarization-induced activation. Similar to S132C, hyperpolarization-induced activation depends upon...
the internal [Cl\(^-\)], and homodimeric mutant channels display a reduced resting open probability at physiological intracellular anion composition. Heterodimeric channels again exhibit a dependence of gating properties on the internal [Cl\(^-\)]. At a physiologically low [Cl\(^-\)]\(_{\text{int}}\), the activation curve is moderately shifted to more positive potentials, reducing the resting open probability by 23%.

All the patients described so far carried the diagnosis of myotonia congenita, but the F428S mutation is responsible for a distinct clinical phenotype. The patient carrying this mutation was diagnosed with paramyotonia congenita (Weiss and Mayer, 1997), a dominantly inherited myotonic syndrome characterized by cold- and exercise-induced exacerbation of symptoms. We and others have documented cold-sensitivity of myotonia congenita patients with the G230E mutation in CLCN1 (Koty et al., 1996). However, in all patients with accompanying muscle weakness reported so far, mutations in SCN4A, the gene encoding the muscle sodium channel, were the cause of disease. We sequenced the whole SCN4A gene and excluded an alteration of the muscle sodium channel as a cause of disease in our patient. Until now, there was a general belief that sodium and chloride ion channel myotonias could be distinguished by their clinical features. Our findings provide additional evidence that mutations in CLCN1 can cause clinical symptoms similar to those seen with SCN4A mutations (Wagner et al., 1998).

F428S causes distinct modifications of chloride channel function. Under both ionic compositions tested, gating properties of F428S homo- or heterodimeric channels are not different from WT. The current amplitudes in cells expressing homo- or heterodimeric F428S channels, however, were significantly smaller compared with cells expressing WT channels. At present, we do not know the molecular basis of the reduced macroscopic current density. Possible reasons are a reduction of the unitary current amplitude, a changed absolute open probability without changes of the voltage dependence of the relative open probability (Fig. 5L; Table 2) or a decreased surface expression because of an impaired translation or intracellular targeting of the mutant muscle chloride channels.

Myotonia congenita represents a group of diseases in which both dominant and recessive inheritance patterns can result from mutations in the same gene (Lehmann-Horn and Jurkat-Rott, 1999). While most mutations described to date have been consistent with either dominant or recessive inheritance of myotonia, some mutations have shown more complex non-Mendelian inheritance patterns (incomplete dominance and variable penetrance) (Koty et al., 1996; Plassart-Schiess et al., 1998). In addition to complicated genetic transmission patterns, the clinical symptoms shown by patients of both dominant and recessive myotonia have shown significant heterogeneity and variable expressivity (Koty et al., 1996). The families reported here also showed evidence of variable expressivity. For L283F, not all family members carrying one mutant allele are symptomatic, suggesting a variable expressivity of certain mutations. For example, the asymptomatic father of patient 2 also carries the L283F mutation. Although WT-L283F and WT-T310M heterodimeric channels display similar alterations of gating, the patient carrying the T310M mutation was almost asymptomatic before and after her pregnancy.

The mutations reported here exhibit novel alterations of muscle chloride channel function and thus significantly increase the number of known functional defects induced by hClC-1 mutations. Until now, only two alterations of CIC-1 function have been reported in Thomsen’s disease. One mutation (G230E) causes an exchange in the presumed CIC-1 selectivity filter and results in prominent changes of ion conduction (Fahlke et al., 1997b). All the other dominant mutations reported so far cause a shift of the activation curve to more depolarized potentials (Pusch et al., 1995b; Beck et al., 1996; Wagner et al., 1998; Zhang et al., 2000b). Each of the novel mutations described here exerts a functional effect that differs from these two prototypic alterations. S132C causes chloride-dependent hyperpolarization-induced activation in homodimeric channels, and depolarization-induced activation with shifted activation in heterodimeric channels. At a high [Cl\(^-\)]\(_{\text{int}}\), L283F exhibits a deactivation defect similar to I329T, a mutation found in a family with recessive myotonia (Zhang et al., 2000b). Despite the similar functional effects in homodimeric channels, L283F exhibits a clear dominant-negative effect in heterodimeric WT-mutant channels. Homodimeric as well as heterodimeric T310M channels have the surprising ability to exhibit normal gating at high internal [Cl\(^-\)], but to display a shifted activation curve at low internal chloride. In T550M homodimeric channels, we observed two separate gating steps, one that activates upon hyperpolarization and one that deactivates upon depolarization. This mutation again causes a right shift of the activation curve of heterodimeric channels only at low internal [Cl\(^-\)]. The variety of functional properties of homo- and heterodimeric channels illustrates that it is impossible to predict the function of heterodimeric channels and thus to distinguish dominant and recessive mutations without testing both channel types under several ionic conditions.

Muscle-type CIC channels are well known to alter gating in response to changes in the external anion composition (Richard and Miller, 1990; Pusch et al., 1995a; Rychkov et al., 1996; Fahlke et al., 1997d). In contrast, changing the internal [Cl\(^-\)] has minor effects on WT CIC-1 gating (Fig. 4). In the past, the effect of altering internal anion composition has been demonstrated only for certain hCIC-1 constructs with inverted voltage-dependence of activation (Fahlke et al., 1995; Zhang et al., 2000a). The finding that several homo- as well as heterodimeric mutant hCIC-1 channels activating upon depolarization exhibit a dramatic shift of the activation curve upon lowering the internal [Cl\(^-\)] provides fresh insights into the function of muscle chloride channels. At present, we are unable to explain this finding mechanistically, but the novel mutations certainly provide a tool to study this physiologically very important dependence. These results also contribute to our understanding of CIC-1 dysfunction in
myotonia. In experiments using a high internal [Cl⁻], several mutations have been reported that exhibit only small changes of gating (Zhang et al., 2000b) that could not account for a significant reduction of $g_{Cl}$ in affected muscle that is necessary to cause myotonia (Adrian and Marshall, 1976). Here we demonstrate the existence of mutant channels that appear quite normal at high internal chloride, but exhibit clear changes of gating at a physiological low internal concentration of permeant anions. It is therefore necessary to test myotonia-causing mutations at a physiological internal [Cl⁻] to gain insights into their specific mechanisms of dysfunction.

In summary, we have tested a large number of myotonic patients for mutations in the CICNI gene and identified six novel disease-causing mutations. Out of these six patients, one patient carried the clinical diagnosis of paramyotonia, demonstrating that chloride channel dysfunction can cause similar symptoms as alterations of sodium channel function. The functional characterization of homo- and heterodimeric mutant channels in a heterologous expression system revealed novel mechanisms of CIC-1 dysfunction causing myotonia. A knowledge of all functional alterations of CIC-1 channels will be a major advance in understanding the molecular pathophysiology of myotonia and will represent an important step towards rational therapeutic strategies. Although myotonia often exhibits a mild phenotype, in certain cases the life-long muscle stiffness can be quite disabling, and novel therapeutic agents could be very beneficial for these patients.

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