

ABSTRACT: Clinical, electrophysiological, and molecular findings are reported for a family with dominant myotonia congenita in which all affected members have experienced long-term fluctuations of the symptom of myotonia. In some patients myotonia is combined with myalgia. The myotonia-causing mutation in this family is in the gene encoding the muscular chloride channel, hCIC-1, predicting the amino acid exchange G200R. We have constructed recombinant DNA vectors for expression of the mutant protein in tsA201 cells and investigation of the properties of the mutant channel. The most prominent alteration was a +100-mV shift of the midpoint of the activation curve. Therefore, within the physiological range the open probability of the mutant channel is markedly smaller than in wild-type. This shift is likely to be responsible for the myotonia in the patients. The fluctuating symptoms of this chloride channelopathy are discussed with respect to short-term fluctuations of myotonia in the sodium channelopathy of potassium-aggravated myotonia.

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THE DOMINANT CHLORIDE CHANNEL MUTANT G200R CAUSING FLUCTUATING MYOTONIA: CLINICAL FINDINGS, ELECTROPHYSIOLOGY, AND CHANNEL PATHOLOGY

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Myotonia congenita is a muscle disorder characterized by a stiffening of the skeletal muscles occurring during voluntary contractions exerted after a period of rest. This symptom is caused by mutations in *CLCN1*, the gene encoding the skeletal muscle chloride channel, CIC-1.¹¹ Both dominant and recessive modes of inheritance are known in myotonia congenita families and the resulting “diseases” are often designated by their discoverer’s name; for instance, Thomsen²⁸ for dominant myotonia congenita (DMC), and Becker² for recessive generalized myotonia (RGM). Intensive research over the past 5 years

has revealed about 30 point mutations and 3 deletions in *CLCN1*. All of these mutations are expected to cause a nonphysiological reduction of the chloride conductance of the muscle fiber membrane.¹⁴ The majority of these mutations result in Becker-type myotonia, and with ongoing research, recessive mutations are still believed to increase in number.^{4,26} In contrast, the number of Thomsen mutations is only four (Table 1), when one excludes Q552R, a dominant mutation causing “myotonia levior,” as well as R894X and G230E, two mutations that cause symptoms to be transmitted as a dominant trait only in some families, whereas in others inheritance is recessive.

As to the severity of myotonia in different patients of a family, there is generally a marked variability. This had already been observed by Becker for all 26 dominant myotonia families he surveyed in his extensive monograph, which also included the Thomsen kindred.² Therefore, a classification of dif-

Abbreviations: DMC, dominant myotonia congenita; hCIC, human muscular chloride channel; HEK, human embryonal kidney; PAM, potassium-aggravated myotonia; PCR, polymerase chain reaction; RGM, recessive generalized myotonia; SSCA, single-stranded conformation analyses.

Key words: muscle disease; chloride; myotonia congenita; *CLCN1* gene; fluctuations.

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Table 1. Shift of midpoint ($V_{0.5}$) of activation curve determined for various dominant mutations in *CLCN1* of man and goat.

Mutant	Expression system	$V_{0.5}$ shift of pure mutant vs. wild-type	Ref.
G200R	X. oocytes	+65 mV	30
	tsA201 cells	+100 mV	29
G230E*	X. oocytes	Nonfunctional	27
	tsA201 cells	-25 mV	6
I290M	X. oocytes	+75 mV	22
R317Q	X. oocytes	+40 mV	22
P480L	X. oocytes	Nonfunctional†	22
Q552R	X. oocytes	Nonfunctional‡	22
R894X*	X. oocytes	ND	19
A885P (goat)	X. oocytes	+47 mV	1

*In some families a recessive mode of inheritance.

† $V_{0.5}$ shift after 1:1 coexpression with WT: +90 mV.

‡ $V_{0.5}$ shift after 1:1 coexpression with WT: +58 mV.

ferent families depending on the degree or severity of affection is difficult, perhaps with the exception of De Jong's myotonia levior.¹³ However, Becker noted some other characteristics of myotonia which he thought consistently present or absent throughout the kindreds investigated by him. Thus, in his monograph,² he used a fluctuating course as one of the leading symptoms for classification of DMC.

With the advent of molecular biology, Ricker and coworkers^{15,24} recognized that, in many of the families with dominant myotonia, the gene defect was localized in *SCN4A*, the gene encoding the α subunit of the muscle sodium channel. Koch¹⁰ subsequently examined the genotype of the families studied by Becker,^{2,3} and found that, among the 11 families still available from the original 26, only 6 had a chloride channel myotonia.

In sodium channel myotonia, the degree of myotonia among different families is particularly variable and, accordingly, the names of myotonia fluctuans,²⁴ acetazolamide-responsive myotonia,²¹ and myotonia permanens¹⁵ were used when the correct phenotype/genotype correlations were first discovered. In contrast to patients with chloride channel defects, in these patients the myotonia was always severely aggravated by the ingestion of potassium. Therefore, all sodium channel myotonias were later subsumed under the name of potassium-aggravated myotonia, PAM.²⁵ It is now clear that the number of families having PAM is far greater than the number of DMC families.¹⁴

Because in some families with potassium-aggravated myotonia the myotonia fluctuates, the question arose as to whether a fluctuating course is a characteristic sign for sodium channel myotonia and is perhaps always absent in chloride channel myoto-

nia. Here we report the clinical findings and electrophysiology of a large family with fluctuating myotonia, for which the genetic defect was clearly localized to *CLCN1*. In addition, we investigated the pathological consequences introduced by the G200R mutation for the chloride channel function by introducing the mutation into a plasmid vector that we expressed in tsA201 cells.

MATERIALS AND METHODS

Patients. The basis of this study was a Turkish family with dominant myotonia (Fig. 1) that, by the use of molecular biology, had been diagnosed as having chloride channel myotonia; that is, the G200R mutation in *CLCN1*.¹⁷ All patients underwent a thorough clinical and electrophysiological investigation.

Molecular Biology. Ten members of this family and more than 100 German and 50 Turkish control probands had genomic DNA extracted from anticoagulated blood. Informed consent was given in all cases. To screen the samples for mutations in *CLCN1*, the DNA was amplified by polymerase chain reaction (PCR) using specific primers for the 23 exons of *CLCN1*.¹³ Single-strand conformation analyses (SSCA) were performed and aberrant bands were investigated by direct PCR sequencing. SSCA of exon 5 revealed an abnormal pattern for the index patient and 5 other family members. All were heterozygous for a missense mutation at cDNA position 598, predicting a substitution of arginine for glycine-200, which is highly conserved among all known members of the *CLCN* gene family. The point mutation



FIGURE 1. Pedigree of the G200R family with polyacrylamide gels displaying PCR-amplified genomic DNA fragments following digestion with *BssHII* and staining with ethidium bromide.

was introduced into the human ClC-1 cDNA using the Altered Sites In Vitro Mutagenesis System (Promega). A 1.4-kb *HindIII*–*SmaI* fragment of hClC-1 was introduced into the pSelect vector and a single-stranded DNA copy was rescued with R408 helper phage. The mutation G200R was constructed using an antisense oligonucleotide (5'-CCC GAA ATG AAG ACA ATA CTA CGT AGG GTT GTC CTG AAG GAA TAC CTC-3'), which contained, in addition, a silent mutation that altered the restriction pattern for *Sna* BI. The oligonucleotide primer was annealed to the single-stranded DNA copy and was extended with T4 DNA polymerase and ligated with T4 DNA ligase. Mutant clones were verified by restriction with *Sna* BI and dideoxynucleotide sequencing. Multiple independent fragments of recombinant mutant clones were subcloned back into the full-length *CLCN1* cDNA pRc/CMV vectors for expression studies.

Transient Transfection of tsA201 Cells. tsA201 cells are a simian virus 40 (SV40) T-antigen-expressing derivative of the human embryonic kidney cell line HEK-293¹⁸ that we preferred because of its increased transfection efficacy. Transient transfection was achieved using the calcium phosphate precipitation method⁹ with ca. 0.01 µg of plasmid DNA per 3–5 × 10³ cells/cm². To detect cells expressing recombinant mutant clones after transfection, the tsA201 cells were cotransfected with a plasmid encoding the CD8 antigen and incubated with polystyrene microbeads coated with anti-CD8 antibody (Dynabeads M-450 CD8, Dynal, Hamburg, Germany) 3 min before electrophysiological characterization.

Oligoclonal HEK-293 cell lines were obtained as described elsewhere.¹²

Patch-Clamp Recordings. Two to 3 days after transfection, standard whole-cell recordings were performed, using an EPC-7 amplifier (List, Darmstadt, Germany). Only cells to which microbeads had bound were used. The standard external solution contained 140 mmol/L NaCl, 4 mmol/L KCl, 2 mmol/L CaCl₂, 1 mmol/L MgCl₂, and 5 mmol/L HEPES (pH 7.4). The recording pipettes were pulled from borosilicate glass, and heat polished. They had resistances between 0.7 and 1.7 MΩ when filled with standard internal solution (130 mmol/L CsCl, 2 mmol/L MgCl₂, 5 mmol/L EGTA, 10 mmol/L HEPES [pH 7.4]). More than 60% of the series resistance was compensated by an analog procedure. Linear leakage or capacitive currents were not subtracted. The currents were filtered using the 3-kHz filter of the amplifier, and sampled at various

rates. For the determination of the electrophysiological properties of G200R channels, we elicited families of chloride currents by applying to the cell membrane the cyclic program illustrated in Figure 2A: from a holding value of 0 mV, the potential was clamped for 400 ms to a variable prepotential, and then to the test potential of –105 mV.

The voltage dependence of activation was determined from such current traces using a combination of three programs (pCLAMP, Axon Instruments, Foster City, CA; Excel, Microsoft, Unterschleissheim,

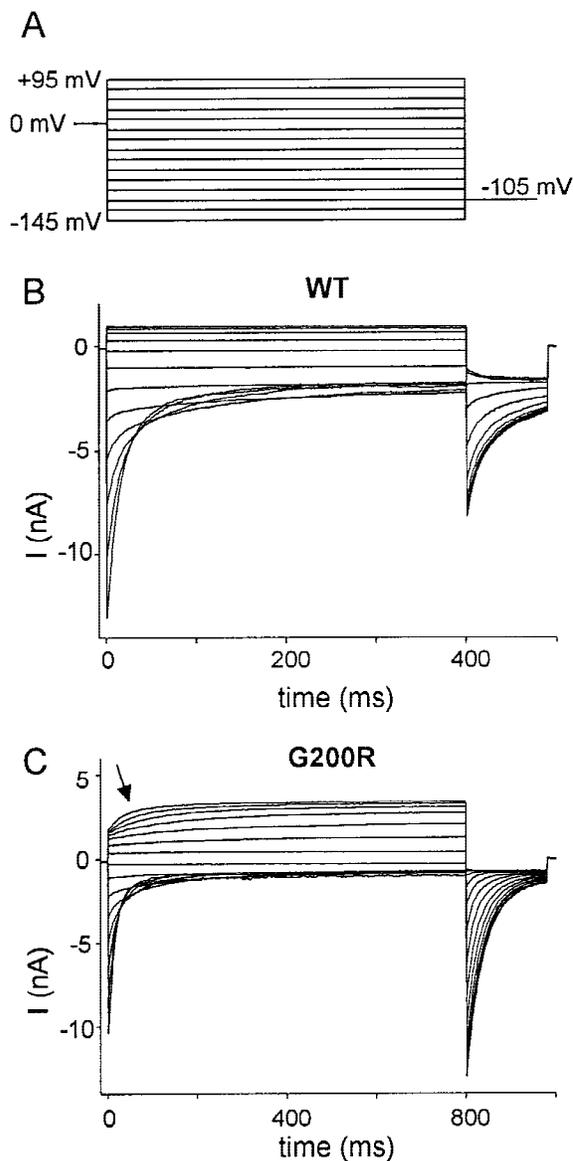


FIGURE 2. Current responses to voltage steps (A) going from a holding potential of 0 mV first to a variable prepotential (varied in 20-mV steps from –145 mV to 95 mV), and then to –105 mV. The instantaneous current amplitudes following the jump to –105 mV are used to determine P_{open} . Wild-type (B) and G200R hClC-1 (C) currents after heterologous expression in tsA201 cells.

Germany; SigmaPlot, Jandel Scientific, San Raphael, CA). The software normalized the instantaneous current amplitudes at the test potential to the extrapolated maximum value and plotted them versus the prepulse potential, yielding the voltage dependence of the relative open probability, P_{open} , at the end of the prepulse.¹²

RESULTS

Clinical Findings. In general, the remarkable findings with this family were: (i) the long-term fluctuation in the symptoms of all affected family members; and (ii) complaints of muscle pain in some of the patients. Medical help was sought only by patient III-2, and it was only with intensive questioning on several occasions that the rest of the affected family members admitted similar complaints. Patients I-2, II-2, and II-5 were clinically and electromyographically examined and they were free of myotonic symptoms and signs. The DNA of 10 family members was studied (Fig. 1). Six of them (I-1, II-1, II-3, II-4, III-1, III-2) showed an aberrant band in SSCA and were positive for the G200R amino acid exchange, consistent with dominant inheritance. The individual clinical findings with the affected family members were as follows.

Patient III-2. Onset of the disease in the 15-year-old index patient was at 2 years of age. He was noted to start walking on his toes, but this became less severe with further walking. A few years later, the stiffness at the onset of a movement had become more noticeable. Stiffness in the hands started at about the age of 7, and stiffness at the onset of talking, chewing, and eye closing at about the age of 9. Mostly involved were the legs, followed by the hands. Other muscles were only occasionally involved. The myotonia in the legs was painful, at times making the patient cry. The fluctuation of symptoms was striking, with symptom-free periods occurring especially in the summer. Cold, hunger, fatigue, and emotional upset had adverse effects. He did not complain of weakness at any time.

Muscle hypertrophy was not observed. Action myotonia was present in the legs and hands. Percussion myotonia was observed in the abductor pollicis brevis, extensor carpi radialis, deltoideus, gastrocnemius, and hypoglossus muscles. He climbed 10 steps in 8 s, improving to 4 s on the third attempt. Strength and reflexes were normal.

Patient III-1. This 16-year-old boy noted, at the age of 10, a stiffness in the legs after a prolonged period of being seated. The stiffness lasted for a few seconds and occurred about once a month. Since the age of 12, he observed occasional stiffness in the

hands, correcting it by himself immediately through a second movement. He has been free of symptoms for about 2 months in winter.

On examination he was found normal except for mild percussion myotonia in the extensor carpi radialis muscle on the right.

Patient II-4. This 28-year-old man first noted stiffness in the hands and legs at the age of 17 after doing folkloric dances for about 8 h. The stiffness continued after this incident without affecting his life to any significant degree. During the previous 2 years, he started experiencing occasional stiffness in the neck and with eye closure. Yawning caused stiffness in combination with a pain below his chin. He felt also some pain connected with the stiffness in the hands and legs. There were symptom free periods that did not last for more than one week, usually in the summer. Cold, hunger, and emotional upset made the clinical findings worse.

On examination, he was found normal except for mild action myotonia on starting to walk. He climbed 10 steps in 10 s, improving to 4 s on the third attempt.

Patient II-3. This 29-year-old man had several very brief episodes of stiffness with pain between the ages of 14 and 18. He had not been aware of any since then. His examination was normal.

Patient II-1. This 34-year-old woman noticed the presence of stiffness during the last trimester of her pregnancy in which she gave birth to patient III-2. She had difficulty when starting to climb stairs and at the beginning of slopes. She also felt stiffening when she came along a small impediment while walking, and she remembers falling down once on such an occasion. In retrospect, she understood that the reason for her hard time during the last months of pregnancy was the stiffness. Mostly affected were the legs followed by the hands, but occasionally there was stiffness in the neck, eyes, and lower face. After the pregnancy, the stiffness occurred once or twice every other week and lasted for a few seconds. There were periods lasting over 1 year where she was not aware of it at all. Cold, extreme hunger, fatigue, and emotional upset had adverse effects.

On examination there was minimal percussion myotonia found in the extensor carpi radialis muscle. The stair test was normal.

Patient I-1. This 60-year-old woman remembered having had a few episodes of stiffness in the legs and upon chewing during some of her pregnancies. She did not recall any stiffness of the hands and eyes at that time. Besides the events during her pregnancies she did not experience any stiffness. Her examination was normal.

Electrophysiological Findings in Patients. All of the aforementioned patients had needle EMGs showing abundant myotonic discharges in all tested muscles. With 2 patients (III-2 and II-4), repetitive nerve stimulation was performed and neither showed a decrement with repetitive nerve stimulation at 5 Hz for 10 s or 10 Hz for 5 s. Single stimuli applied after 10 s of exercise showed a 10% decrement in patient II-4. This is consistent with the diagnosis of DMC.

Properties of G200R Channels Expressed in Mammalian Cells. When we recorded the currents from tsA201 cells transfected with either wild-type or G200R hClC-1 DNA, we observed currents not seen in untransfected cells (Fig. 2). The currents with wild-type hClC-1 (Fig. 2B) were virtually identical to those observed with stably transfected HEK-293 cells¹²; therefore, we felt entitled to use some of our earlier data obtained with HEK-293 cells as controls. The current amplitudes (and thus the level of channel expression) seen with the G200R mutant (Fig. 2C) resembled those observed with wild-type channel. For both channels, the measured current reversal potential was nearly identical to the calculated chloride equilibrium potential (-2.8 mV). The currents were blocked by external application of the chloride channel blocker 9-anthracene carboxylic acid ($100 \mu\text{mol/L}$).

Wild-type hClC-1 is characterized by a rapid but incomplete deactivation upon hyperpolarization (Fig. 2B) and by pronounced inward rectification of the instantaneous currents.⁸ G200R resembled wild-

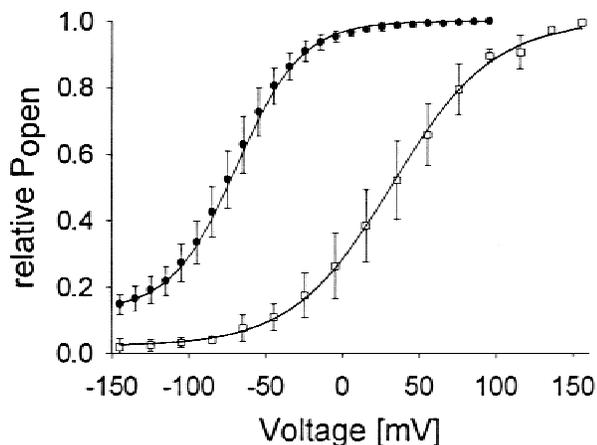


FIGURE 3. Voltage dependence of the relative open probability P_{open} for WT (filled circles; $n = 5$) and G200R (empty squares; $n = 4$) channels expressed in HEK-293 and tsA201 cells, respectively. Activation curves were fitted to the data using a single Boltzmann term and a voltage-independent value: $I(V) = A\{1 + \exp[(V - V_{0.5})/k_v]\}^{-1} + \text{constant}$ (means \pm SD).

type in these properties, although with the slight modification that, at positive potentials, current activation was time-independent in WT, whereas the mutant exhibited activation (arrow in Fig. 2C). To reach steady-state levels, the prepotential pulses were therefore prolonged to 800 ms in the case of G200R.

The activation curve of G200R was drastically altered (Fig. 3). Whereas the fraction of open wild-type channels increased as the membrane potential went positive from -100 mV ($P_{\text{open}} = 0.25$) to about -20 mV ($P_{\text{open}} = 1$), the population of open mutant channels only began to increase substantially at -10 mV ($P_{\text{open}} = 0.25$) and did not reach $P_{\text{open}} = 1$ at membrane potentials less than $+100$ mV. Fits of Boltzmann functions to these data yielded half-maximum P_{open} to be at -69.2 ± 7.5 mV ($n = 5$) for the wild-type channel and at $+33.1 \pm 17.1$ mV ($n = 4$) for G200R. Thus, the midpoints of the two activation curves were 102 mV apart. Also, the slope factors, k_v , determined at the midpoints, were significantly different (-31.0 ± 3.2 mV for G200R vs. -20.8 ± 0.9 mV for wild-type, $P < 0.05$, t -test).

DISCUSSION

To our knowledge, this family is the first DMC kindred for which the phenotype of fluctuating myotonia has been correlated without doubt to the genotype of chloride channelopathy. One of the striking findings with the members of this family was the fact that many of them had symptom-free periods for weeks to months. This is what Becker had also observed in some of the families he studied. Thus, we have presented clear evidence that there are kindreds with the chloride channelopathy of dominant myotonia congenita (DMC) with fluctuating myotonia. Another family presenting fluctuating myotonia—although less pronounced—carried the *CLCN1* mutation predicting I290M. In other kindreds (e.g., Thomsen's own family), myotonia never was found to fluctuate.

This fluctuation of symptoms in DMC is, however, different from the day-to-day fluctuations characteristic for the sodium channelopathy of potassium-aggravated myotonia (PAM), in particular for the variant of myotonia fluctuans. In the words of Becker, the fluctuations in DMC are "periods of marked disturbance that alternate with periods of less pronounced symptoms in which the patients are less impeded or in which they feel almost or completely free. These phases last years or months, seldom weeks or days."² This contrasts with the description of myotonia fluctuans by Ricker and coworkers: There, myotonia presents with "occasional episodes of intermittent generalized myotonic stiffness" . . .

“The patients with myotonia were also very sensitive to potassium challenge.”²³

Another of Becker's² leading symptoms was myalgia. We did not find an explanation for the occasional muscle pain reported by some of our patients, in particular by the *propositus*. In our experience, myalgia is reported every now and then with myotonia patients and we do not know the reason for it, nor could we correlate it with particular mutations. Additional circumstances, such as nutrition, may play a role.

The major alteration of the channel parameters of the mutant G200R was a parallel shift of the channel activation curve along the voltage axis by about +100 mV. The chloride channel is most probably a homomeric dimer of ClC-1.^{7,16,20} Thus, in the patients, the functional channels are likely to be made up of 25% wild-type dimers, 25% G200R dimers, and 50% wild-type/G200R bastards. The resulting right-shift of the overall curve with this mixture is probably only about half as large.^{22,30} Nevertheless, in a patient, more than 80% of the channels would be closed at the resting potential of skeletal muscle (-75 to -80 mV), in contrast to about 50% in unaffected persons. The resulting chloride current would not sufficiently stabilize the membrane potential following repolarization, and this could give rise to myotonia. Whether the observed changes are caused by a direct influence on the gate or rather by a shift of the energy levels of closed and open states remains to be elucidated.

An activation curve that is shifted in the positive direction is a common finding for the mechanism of a reduced chloride conductance in dominant myotonia congenita (6 of 7 cases investigated, including myotonia in the goat; see Table 1). Most of the investigations of dominant mutants of *CLCN1* were performed with the channels expressed in *Xenopus* oocytes,^{19,22,27,30} and in our experience the shifts obtained in different expression systems were not easily comparable. Moreover, the values obtained with oocytes were confounded with the problem that some mutants were only measurable when coexpressed with wild-type channels, which should influence the shift of the activation curve in a way that is not exactly predictable. Thus, before more mutants are expressed in mammalian cells, it is probably better to refrain from drawing conclusions from the amplitudes of the various shifts (see Table 1). Nevertheless, we might mention that the Thomsen mutation, P480L, expressed in oocytes, produced a shift of +90 mV, although the expression was at a 1:1 ratio with wild-type ClC-1 channels. Thus, in the Thomsen kindred, where the myotonia did not fluctuate, the shift

seems particularly large. On the other hand (and with the reservations that have to be made for a comparison between man and goat myotonia), the goat mutation, A885P, when expressed by itself in oocytes, produces a right-shift of the activation curve of only +47 mV. Bryant noted that myotonia in the goat may fluctuate a great deal.⁵ More experiments will be needed before a decision can be made whether or not the stability of the symptom of myotonia is correlated with the amount of right-shift of the activation curve produced by a particular dominant mutation.

The results obtained with this family show that the clinical classification made by Becker² for dominant myotonia congenita still has some value, as some families show fluctuation of their myotonia and others do not. Most families with “fluctuating” myotonia reported so far have had sodium channelopathy of PAM.

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