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# Mutations in Dominant Human Myotonia Congenita Drastically Alter the Voltage Dependence of the CIC-1 Chloride Channel

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## Summary

**Autosomal dominant myotonia congenita (Thomsen's disease) is caused by mutations in the muscle chloride channel CIC-1. Several point mutations found in affected families (I290M, R317Q, P480L, and Q552R) dramatically shift gating to positive voltages in mutant/WT heterooligomeric channels, and, when measurable, even more so in mutant homooligomers. These channels can no longer contribute to the repolarization of action potentials, fully explaining why they cause dominant myotonia. Most replacements of the isoleucine at position 290 shift gating toward positive voltages. Mutant/WT heterooligomers can be partially activated by repetitive depolarizations, suggesting a role in shortening myotonic runs. Remarkably, a human mutation affecting an adjacent residue (E291K) is fully recessive. Large shifts in the voltage dependence of gating may be common to many mutations in dominant myotonia congenita.**

## Introduction

Myotonia congenita, or Thomsen's disease (Thomsen, 1876), is an autosomal dominant disorder that affects muscle relaxation. A closely related autosomal recessive disease is called recessive generalized myotonia, or Becker type myotonia (Becker, 1977). Patients with myotonia experience muscle stiffness after voluntary contraction that gradually gets better with exercise (the so-called "warm-up" phenomenon; for review, see Rüdel and Lehmann-Horn, 1985). Myotonia is due to an electrical instability of the muscle membrane itself, leading to series of repetitive action potentials with a single stimulus ("myotonic runs"). The pioneering studies of Bryant and colleagues (Lipicky and Bryant, 1966; Lipicky et al., 1971) revealed a decrease in muscle membrane chloride conductance in both animal models and patients. This was confirmed in later studies (Rüdel et al., 1988; Franke et al., 1991). In fact, the major skeletal muscle chloride channel CIC-1 (Steinmeyer et al., 1991b) is inactivated in the recessive myotonic mouse strain ADR (Steinmeyer et al., 1991a), and both dominant and recessive forms of human myotonia are due to mutations in this gene (Koch et al., 1992; George et al., 1993; Steinmeyer et al., 1994). More than 20 different CIC-1 mutations have now been identified in patients with recessive or dominant myotonia (George et al., 1994; Heine et al., 1994; Lorenz et al., 1994; Meyer-Kleine et al., 1994, 1995; Lehmann-Horn et al., 1995; for review, see Jentsch et al., 1995b).

The mechanism by which a decrease in muscle chloride conductance leads to myotonia is easily understood. Under resting conditions, chloride accounts for 70%–80% of muscle membrane conductance, stabilizing the voltage at the chloride equilibrium potential. Thus, chloride conductance contributes significantly to the repolarization of action potentials. In its absence, sodium channels have enough time to recover from inactivation while the membrane potential is still depolarized, leading to the typical myotonic runs. These series of repetitive action potentials subside after some time ("warm-up"). This may be due to the activation of other ion channels caused by changes in the intracellular milieu during these periods of hyperactivity.

Both dominant and recessive myotonia are due to mutations in the same gene, CIC-1. Dominant myotonia cannot be due to haploinsufficiency, as a total loss of functional chloride channels on only one allele does not lead to myotonia (Steinmeyer et al., 1991a; Gronemeier et al., 1994; Meyer-Kleine et al., 1994). It has been shown that dominant myotonia is due to dominant negative effects of mutant subunits on wild-type (WT) subunits coexpressed in muscles of heterozygous patients. Coexpression in Xenopus oocytes using two different dominant CIC-1 mutations strongly suggested that CIC-1 functions as a homomultimer with at least three subunits (Steinmeyer et al., 1994).

In this work, we analyze several dominant mutations in CIC-1 and show that their voltage dependence of gating is drastically changed. Heterooligomeric mutant/WT channels display an intermediate shift along the voltage axis that is still large enough to explain myotonia in heterozygous patients. As we found similar effects with several different mutations, this may be the single most common mechanism underlying mutations in dominant myotonia.

## Results

Using single-stranded conformation polymorphism analysis, we identified a CIC-1 mutation (I290M) cosegregating with the disease in a pedigree with typical dominant myotonia congenita (Figure 1A) (Koty et al., 1994; Lehmann-Horn et al. 1995). Isoleucine 290 is located at the end of putative transmembrane domain D5 in a highly conserved stretch (Figure 1C). Sequence comparison suggests that only hydrophobic residues are allowed at this position, as other CIC proteins have either isoleucine or leucine at the equivalent position. The glutamic acid immediately following I290 is even conserved in all known members of this

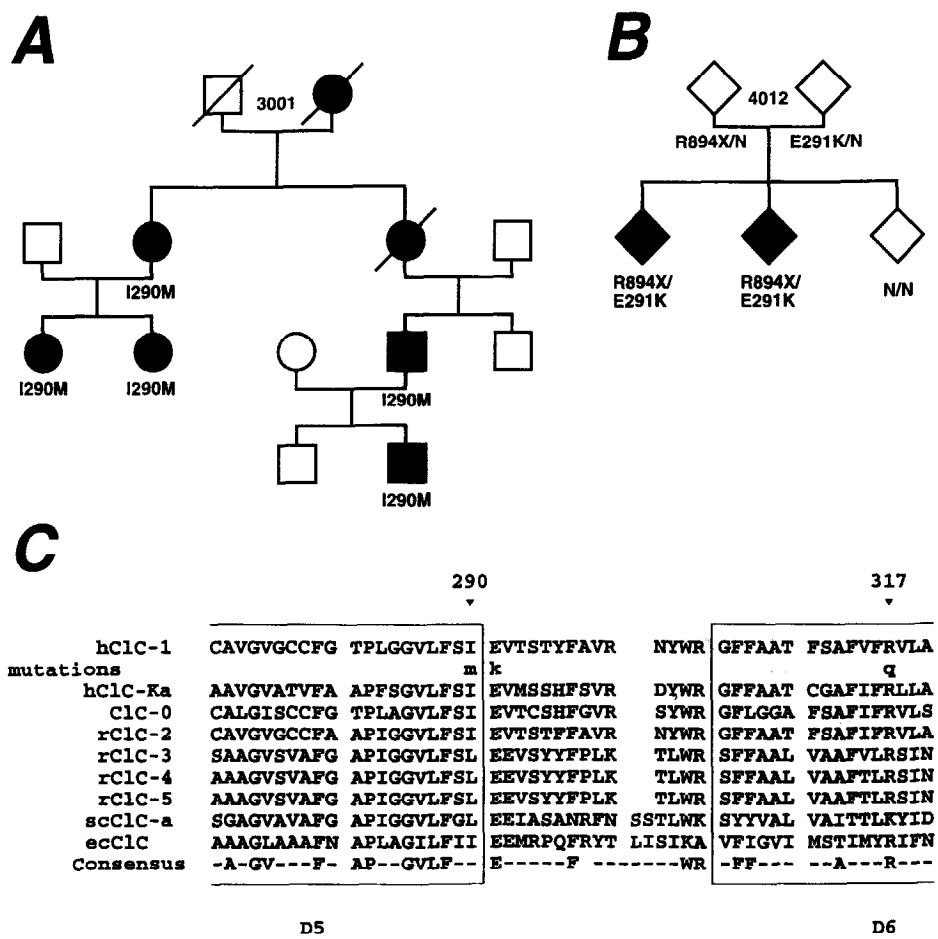


Figure 1. Dominant and Recessive Myotonic Mutations Found in Two Different Families

Mutation analysis in pedigrees showing dominant myotonia congenita (A) and recessive generalized myotonia (B). (C) alignment of homologous ClC proteins in the region of putative transmembrane domains D5 and D6. The isoleucine at position 290 that is mutated to methionine in the dominant pedigree is the direct neighbor of the glutamate 291 mutated to lysine in the recessive family. In the recessive family, the mutation on the other allele is R894X, which may be dominant in some families (George et al., 1994), but is clearly recessive in others (Meyer-Kleine et al., 1995). Squares indicate males, and circles females. For the sake of anonymity, sexes have not been indicated in the recessive pedigree. Affected family members are indicated by closed symbols. Also shown is mutation R317Q, which was found in another pedigree with dominant myotonia (Meyer-Kleine et al., 1995). hClC-Ka is a human kidney-specific ClC protein (Kieferle et al., 1994); ClC-0, the chloride channel from Torpedo electric organ (Jentsch et al., 1990); ClC-2 is a ubiquitous swelling-activated chloride channel from rat (Thiemann et al., 1992); ClC-3 and ClC-4 are rather broadly expressed ClC proteins (Kawasaki et al., 1994; van Slegtenhorst et al., 1994; Jentsch et al., 1995a); ClC-5 is predominantly expressed in kidney (Fisher et al., 1994; Steinmeyer and Jentsch, unpublished data); scClC-a is the S. cerevisiae ClC protein GEF-1 (Greene et al., 1993); and ecClC is the predicted product of an open reading frame of E. coli (Fujita et al., 1994). The prefix r in front of ClC means rat and h, human. Putative transmembrane domains D5 and D6 are boxed, and mutations found in myotonic families are indicated.

gene family, including gene products from S. cerevisiae (Greene et al., 1993) and E. coli (Fujita et al., 1994). Interestingly, a mutation at this neighboring amino acid (E291K) was identified in a pedigree with recessive myotonia of the Becker type (Figure 1B) (Meyer-Kleine et al., 1995). Thus, human missense mutations at these two adjacent residues differ totally in their mode of inheritance.

To gain insights into the mechanism of these mutations, we introduced them into the human ClC-1 cDNA (Steinmeyer et al., 1994) and performed functional analysis in Xenopus oocytes. Figure 2 shows voltage-clamp traces of oocytes expressing WT ClC-1 (Figure 1B) and the I290M mutant described above (Figure 2C). For comparison, panels D–F of Figure 2 show results obtained with other dominant ClC-1 mutants (in part, coinjected with WT ClC-1), which will be discussed in more detail below. Wild-type ClC-1 (Figure 2B) is closed by a gating process that operates in the 10 ms range and that closes the channel upon hyperpolarization, resulting in the typical maximum of steady-state inward currents at about −100 mV (Steinmeyer et al., 1991b). Gating can be described by a Boltzmann distribution with half-maximal open probability ($p_{open}$) at ≈ −20 mV and a nominal gating charge of ~0.8 (Figure 3B). The dominant mutation I290M did not abolish chloride currents (Figure 2C). Its gating, however, was altered drastically. The open probability is shifted by about 75 mV to more positive potentials, and the nominal gating charge was nearly unchanged (0.9). While wildtype ClC-1 is 15% open at the resting voltage of skeletal muscle (−70 to −80 mV), I290M is nearly totally closed under these conditions (Figure 3B). Thus, I290M cannot contribute to the repolarization of an action potential.

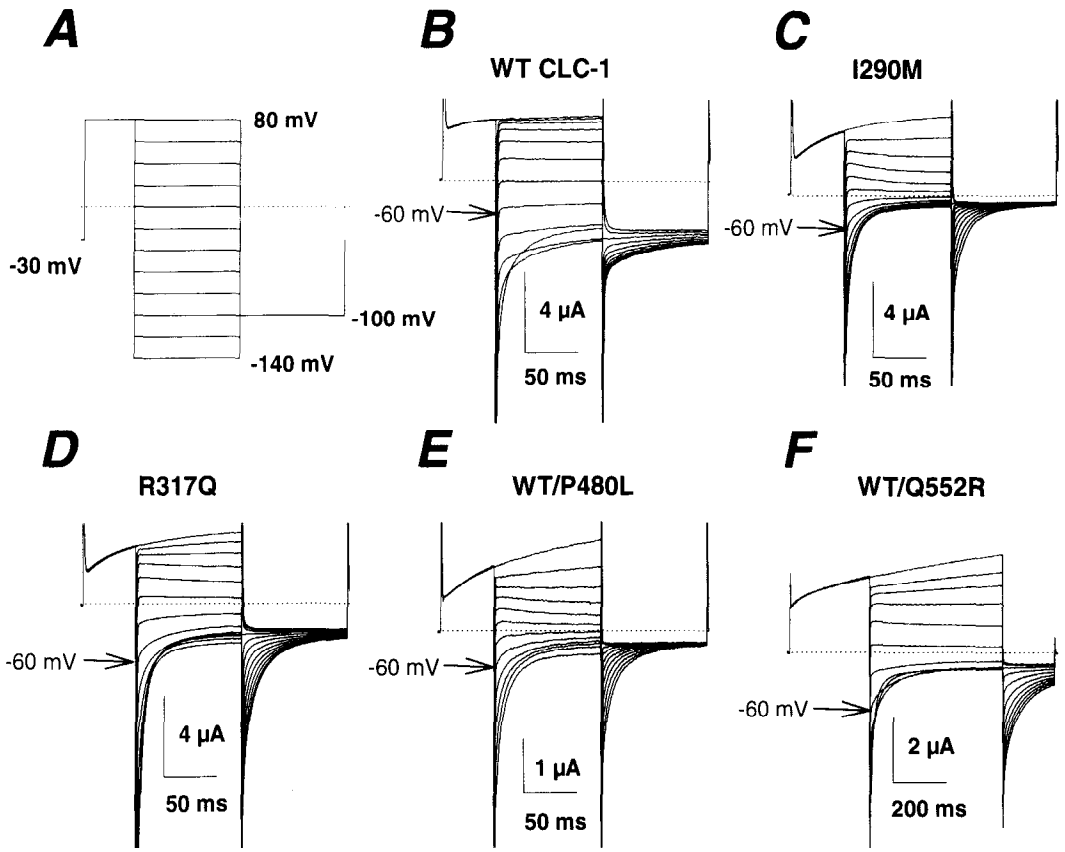


Figure 2. Electrophysiological Characteristics of Dominant Myotonic Mutations

Comparison of currents mediated by WT ClC-1 (B) and four mutants leading to dominant myotonia in humans (C–F). Currents of Xenopus oocytes expressing these channels were measured by two-electrode voltage-clamp technique. (A) Pulse protocol; starting from a holding potential of −30 mV, the voltage was first clamped to +80 mV, which completely opens the gate of WT ClC-1. Voltage was then clamped for 100 ms to voltages between +80 and −140 mV in steps of 20 mV. For the experiment with WT/Q552R coinjection, the pulse lengths were increased to 400 ms, since this heteromeric channel gates slowly. For mutants P480L and Q552R, we used coinjections with WT (E and F), since homomeric mutant channels do not yield currents in this voltage range.

This demonstrates that I290M will cause myotonia in a patient homozygous for that mutation or in compound heterozygotes carrying a null mutation on the second allele. The medically relevant situation with this dominant mutation, however, is coexpression with WT alleles. When I290M was coexpressed with WT ClC-1 at a 1:1 concentration ratio, $p_{open}$ was shifted by approximately one-half the voltage as compared to I290M (i.e., by 45 mV as compared to 75 mV; Figures 3A and 3B). The slope of $p_{open} = f(V)$ was nearly unchanged, yielding again a gating charge of 0.9. This excludes the possibility that the intermediate shift is just due to the superposition of currents of independent, coexpressed WT and I290M channels, because this would lead to a less steep voltage dependence of macroscopic currents (Figure 3B, dashed curve). Thus, WT/I290M heterooligomers are formed in which the mutant subunits partially impose their shifted voltage dependence on the channel complex. This shift is large enough to prevent these channels from contributing to the repolarization of single action potentials. This novel mechanism fully explains why I290M causes dominant myotonia.

To explore the relationship between structure and function at this position, we replaced I290 by 18 different amino acids (Figure 3C). When I290 was mutated to some amino acids (N, H, P, S, A, R, and D), we could not detect currents above background. With the exception of I290V, in which gating is shifted by -17 mV, all other replacements shifted $p_{open}$ to more positive voltages. These shifts ranged from +30 mV for I290C to +115 mV for I290K. Thus, most mutations at this residue would lead to myotonia. If, as a rule, mutant/WT heteromers display an intermediate shift in $p_{open}$, we predict that I290C would behave as a recessive mutation. Mutants I290E, I290L, and I290Q may be either recessive or dominant, possibly depending on the genetic background as with other ClC-1 mutations (Steinmeyer et al., 1994; Meyer-Kleine et al., 1995). It seems difficult to discern a clear-cut pattern from our mutational analysis. There is no relationship to simple parameters like charge or hydrophobicity.

ClC-1 was suggested to function as a tetramer (Steinmeyer et al., 1994). This would allow for different stoichiometries of mutant/WT heterooligomers. When we systematically varied the ratio of mutant I290M to WT

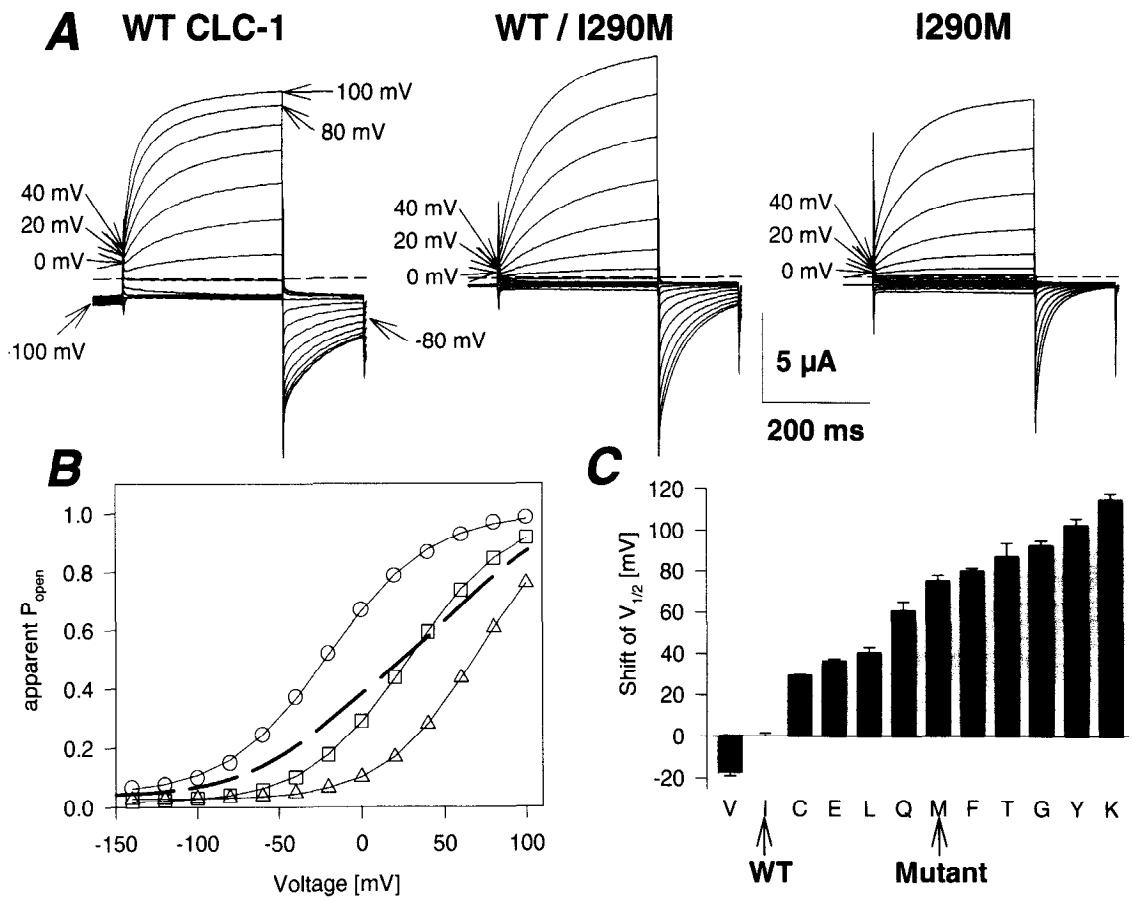


Figure 3. Functional Characterization of the Dominant ClC-1 Mutant I290M and of Other Mutations at the Same Position

(A) Wild-type (WT) human ClC-1 cRNA and mutant I290M cRNA were injected into Xenopus oocytes either alone or in a 1:1 mixture, which mimics the situation in a patient heterozygous for that mutation. Oocytes were examined by two-electrode voltage clamping after two days, yielding the current traces shown. The pulse protocol used a holding potential of −100 mV (close to the resting voltage of skeletal muscle). The oocyte was then sequentially clamped for 300 ms to voltages between +100 mV and −140 mV in steps of 20 mV. (B) Apparent open probability $p_{open}$ as deduced from tail currents at −80 mV, as described in the Experimental Procedures; (circles) WT ClC-1, (squares) 1:1 coinjection of WT and I290M ClC-1, (triangles) I290M ClC-1. The shifts in $p_{open}$, as calculated by fitting Boltzmann equations to these experiments, were 45 ± 6 mV (SD, n = 5) for the 1:1 coinjection and 75 ± 7 mV for I290M by itself. The values for the apparent gating charges, z, obtained from these fits are 0.82, 0.84, and 0.91, respectively. The dashed curve is calculated by averaging $p_{open}$ of WT ClC-1 and I290M ClC-1. The resulting shallow slope is inconsistent with the data obtained from the coexpression experiment, demonstrating the formation of WT/I290M heterooligomers with a shifted voltage dependence of gating. (C) Shifts in voltage-dependent gating of ClC-1 caused by various replacements of the isoleucine at position 290. The half-maximal voltage $V_{½}$ for the opening of the ClC-1 gate was obtained by Boltzmann fits. Mutants in which I290 was replaced by N, H, P, S, A, R, and D could not be expressed. Most other replacements resulted in a shift toward more positive potentials, the only exception being I290V. For each mutation, the mean value of at least 8 oocytes is shown. Error bars indicate SEM.

cRNA, we observed a steady shift in $p_{open}$ = f(V) (Figure 4A). Both the nominal gating charge and the peak current remained about constant (Figures 4B and 4C). Unfortunately, model calculations showed that these experiments do not allow conclusions to be drawn on channel stoichiometry. For instance, within the experimental error, our experiments are compatible with a tetramer in which WT subunits can be replaced by mutant ones in any combination, leading to a shift in voltage dependence that is proportional to the number of mutant subunits incorporated; the data are also equally well explained by a dimeric channel model, which, however, is excluded by an earlier study (Steinmeyer et al., 1994).

With both mutant homomers and mutant/WT heteromers, we could not detect significant changes in the properties of the open pore. Ion selectivity and the voltage dependent block by iodide (Pusch et al., 1994; Steinmeyer et al., 1994) were unchanged (data not shown).

In muscle, the inability of WT/I290M heteromers to repolarize action potentials will lead to the repetitive action potentials ("myotonic runs") typical of myotonia. Although a single action potential is too short to significantly voltage-activate the heteromer, we wondered whether myotonic runs could lead to a slow, incremental activation of these channels. This could shorten the duration of myotonic runs. We mimicked myotonic runs in the oocyte by a 66 Hz train of 5 ms pulses to +40 mV from a holding potential of −80 mV. Although such tetanic stimulation does not normally occur in healthy muscle, we first investigated the effect on WT ClC-1 (Figure 5A). Within 600 ms, chloride conductance activated significantly to a new steady-state value. Starting from $p_{open} \approx 15\%$ at rest (−80 mV), its value

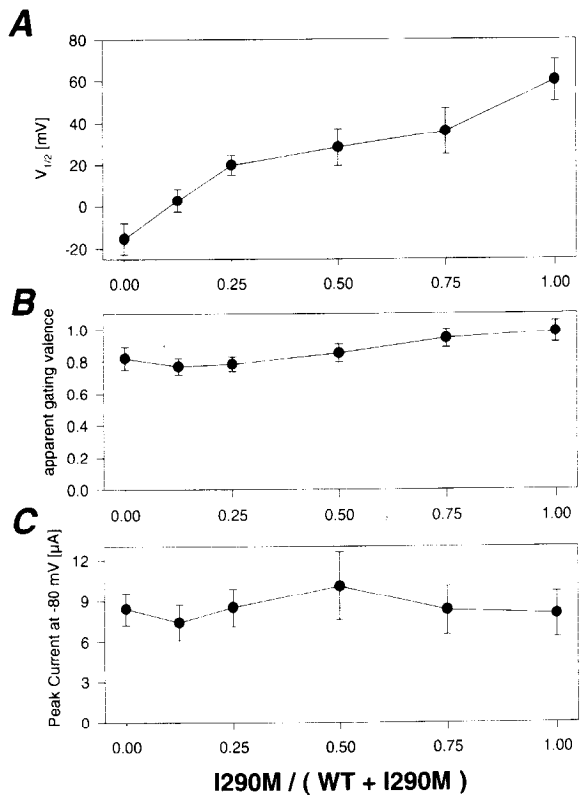


Figure 4. Functional Analysis of WT and I290M ClC-1 Proteins Expressed at Different Ratios

At constant cRNA concentrations, Xenopus oocytes were injected with mixtures of both RNAs. Using voltage clamp protocols and Boltzmann fits as in Figure 3, the voltage $V_{½}$ for half-maximal activation of the gate (A), the apparent gating valence (B), and the peak current at −80 mV (C) were obtained. Voltage dependence of gating shifts steadily to positive voltages with the amount of mutant I290M injected. The gating valence and the peak current remain approximately constant, demonstrating the formation of heteromeric channels having identical open channel conductances. Peak current is the absolute magnitude of the peak inward current at −80 mV after voltage activation at +100 mV and serves as an estimate for the number of functional channels. Data points were averaged from 5 oocytes each, and error bars indicate SD. Similar results were obtained in two other independent experiments.

increases to about 60%. Although the absolute increase in $p_{open}$ with the same pulse program is less with the WT/I290M heteromer (Figure 5B), the relative increase is substantial. $p_{open}$ increases from 0%–5% to 18%, which is close to $p_{open}$ of WT ClC-1 under resting conditions. Thus, we suggest that myotonic runs will be shortened in WT/I290M patients in comparison to patients having two null alleles.

We next investigated the effects of the mutation found in the neighboring residue in the recessive pedigree shown in Figure 1B. E291K ClC-1 did not yield chloride currents in the oocyte between −140 and +100 mV, suggesting that it totally abolishes channel activity. We also investigated whether it interacts with WT subunits (Figure 6A). Coinjections at a 1:1 ratio mimic the situation in heterozygous patients. Noninteracting (recessive) mutants are expected to yield about 50% of WT currents when the same amount of total RNA is injected (Lorenz et al., 1994). As controls, we included the dominant P480L mutant (found in Dr. Thomsen's own family; Steinmeyer et al., 1994), and the recessive R496S mutant (Lorenz et al., 1994). As described previously, the dominant P480L mutant strongly reduced currents in the physiological voltage range. In contrast, currents observed with R496S/WT or E291K/WT coinjections were about 50% of WT, consistent with the fact that E291K is inherited recessively. E291K/WT coinjections did not significantly shift the voltage dependence of WT ClC-1 (Figure 6B), again compatible with a lack of interaction.

Since reversing the charge of E291 is expected to have drastic effects, we also introduced more subtle changes at this position. Conserving the negative charge by replacing glutamate with aspartate (E291D) indeed yielded functional channels. In contrast to WT, and similar to most mutations at I290, gating was shifted to positive potentials (Figure 6B). This shift (27 mV) seems large enough to cause myotonia when found in patients. Replacing the charge by a hydrophobic amino acid (E291L) again destroyed channel activity. Hence, a negative charge seems essential at this position, and conservative mutations drastically affect gating.

Thus, the I290M mutant causes dominant myotonia by imposing a large shift in the voltage dependence of gating on the heteromeric WT/mutant channel. Further, many replacements at this position have similar effects, as has the E291D mutation at the neighboring position. We next asked whether shifts in the voltage dependence are also found with other mutations causing Thomsen's disease.

R317Q, a dominant ClC-1 mutation identified recently (Meyer-Kleine et al., 1995), indeed shows similar effects (see Figure 2D). $p_{open}$ of homooligomeric R317Q channels is shifted by about 40 mV along the voltage axis (Figure 6B), and a 1:1 coinjection with WT ClC-1 yields a shift of +25 mV (data not shown), which is enough to cause myotonia. In both cases, the nominal gating charge remained constant at 0.8.

R317 is located within putative transmembrane domain D6 and is conserved in all known mammalian ClC proteins. In the yeast homologue scClC-a (Greene et al., 1993), it is replaced by a lysine (see Figure 1C). It is surprising that neutralizing this highly conserved charge has relatively minor effects, but the equivalent mutation (R252Q) in the Torpedo channel ClC-0 even yields wild-type currents (Pusch et al., 1995).

P480L, the mutation present in Dr. Thomsen's (Thomsen, 1876) own family, does not yield currents in oocytes and exerts a strong dominant negative effect (Steinmeyer et al., 1994). In that work, we did not examine the very positive voltages where large shifts of $p_{open}$ become apparent. Re-examination of P480L did not reveal currents even at +100 mV. However, we discovered a large shift in the gating of WT/P480L heteromers (see Figure 2E). $p_{open}$ was shifted by about 90 mV to positive voltages, a shift as large as with I290M homooligomers. If one assumes that this shift is approximately one-half of that inherent to the mutant homomer, gating of P480L may be shifted by 180 mV.

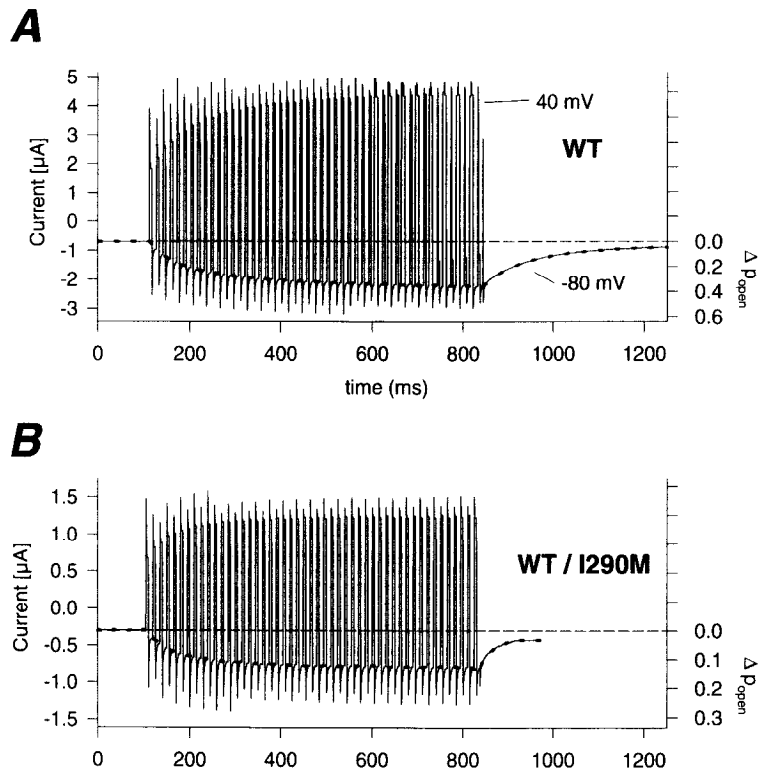


Figure 5. Simulation of "Myotonic Runs"

Repetitive short depolarizations lead to an incremental activation of WT ClC-1 (A) and WT/I290M heteromeric ClC-1 (B) expressed in Xenopus oocytes. From a holding potential of −80 mV, a 66 Hz train of 5 ms pulses to +40 mV was applied. Left scale shows currents, while the right scale gives the increase in open probability elicited by this protocol. The experiments show a significant increase in open probability also for WT/I290M heteromeric channels, suggesting that they may contribute to the temporal limitation of myotonic runs in patients. Increase in $p_{open}$ (dashed line, referring to right scale) was obtained by appropriate scaling of the current at −80 mV with the current flowing after maximum stimulation as measured in the same oocyte.

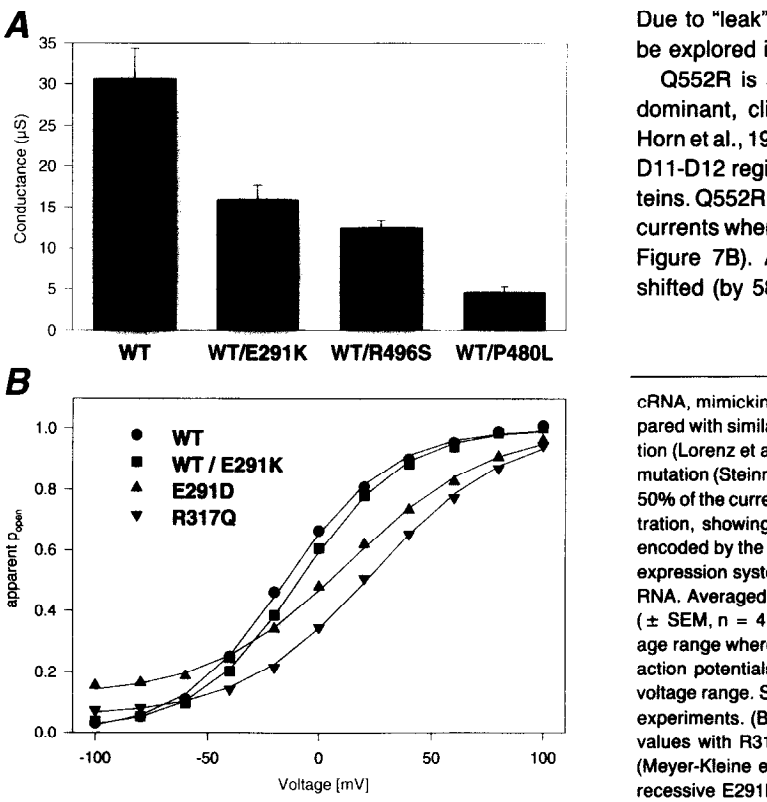


Figure 6. Functional Characteristics of Mutations E291K, E291D, and R317Q

(A) In vitro analysis confirms that E291K acts as a recessive mutation. E291K cRNA was coinjected at a 1:1 concentration ratio with WT ClC-1 cRNA, mimicking the situation in a heterozygous patient. This is compared with similar coinjections using R496S, a known recessive mutation (Lorenz et al., 1994), and to P480L, a strongly dominant negative mutation (Steinmeyer et al., 1994). WT/E291K coinjection elicits about 50% of the current evoked by WT ClC-1 at the same total RNA concentration, showing that it does not interfere with the channel subunits encoded by the WT cDNA. Problems with the saturation of the oocyte expression system are avoided by injecting identical amounts of total RNA. Averaged steady-state slope conductance at −30 mV is shown (± SEM, n = 4 oocytes each). This represents the physiological voltage range where chloride channels contribute to the repolarization of action potentials. P480L/WT channels are not fully activated in this voltage range. Similar results were obtained in two other independent experiments. (B) The apparent open probability is shifted to positive values with R317Q found in a dominant myotonia congenita family (Meyer-Kleine et al., 1995) and the E291D mutant, but not with the recessive E291K mutant coinjected with WT ClC-1. This coinjection is necessary since E291K is nonfunctional by itself. Boltzmann fits to these curves yielded the following values for the half-maximal voltage $V_{½}$ and the nominal gating charge z, respectively: WT, −14 mV and 1; WT/E291K, −8 mV and 1; E291D, +13 mV and 0.8; R317Q, +24 mV and 0.9.

Due to "leak" currents, this voltage range can no longer be explored in Xenopus oocytes.

Q552R is a ClC-1 mutation found in a pedigree with dominant, clinically rather benign myotonia (Lehmann-Horn et al., 1995). This glutamine in the broad hydrophobic D11-D12 region is conserved in some but not all ClC proteins. Q552R was nonfunctional by itself, but elicited novel currents when coexpressed with WT ClC-1 (Figure 2F and Figure 7B). Again, voltage activation was dramatically shifted (by 58 mV) to positive potentials (Figure 7C). In

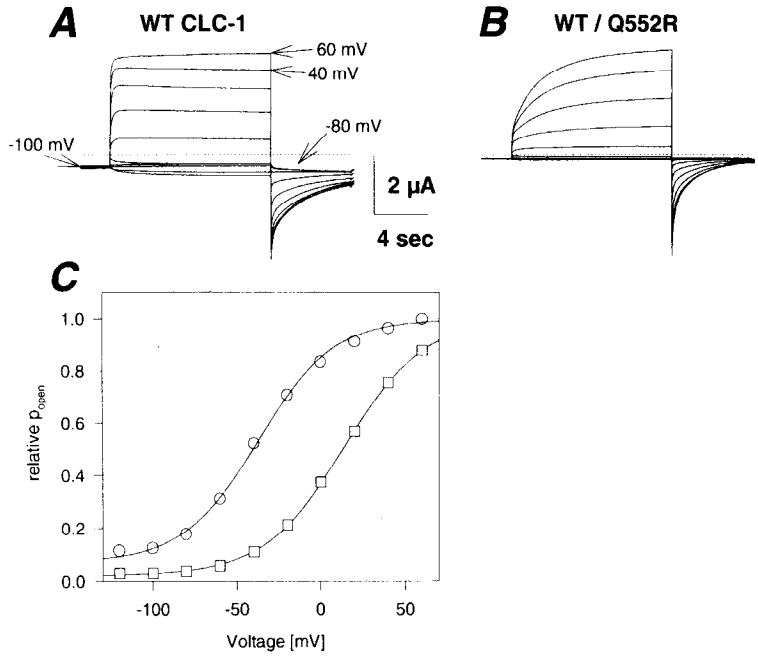


Figure 7. Functional Analysis of the Dominant Mutation Q552R

The mutation Q522R was found in families with rather benign myotonia (Lehmann-Horn et al., 1995). As the Q552R mutant did not yield currents by itself, WT ClC-1 (A) was compared with currents elicited by WT/Q552R coinjection (B). Starting from a holding potential of −100 mV, the oocytes were clamped sequentially to values between +60 and −140 mV in steps of 20 mV for 12 s each. Note the shift in voltage-dependence and the large difference in kinetics. (C) Relative open probability $p_{open}$ for WT ClC-1 (circles) and WT/Q552R heteromers (squares). $p_{open}$ was estimated from tail currents as shown in (A and B), and fitted by Boltzmann equations (lines). When such fits from 5 independent experiments were averaged, they gave half-maximal voltages $V_{½}$ for activation of −39 ± 3 mV for WT, and +19 ± 14 mV for WT/Q552R heteromers. With these long pulses, more negative values of $V_{½}$ than with our usual pulse protocol (see Figure 3) are obtained due to the contribution of a very slow gating component.

contrast to the other dominant ClC-1 mutations, however, there was also a conspicuous change in gating kinetics (cf. Figures 7A and 7B). Strong depolarizations opened the channel very slowly, and activation did not reach steady state even after 12 s. The large voltage shift, combined with the drastically slower gating, suggests that Q552R will behave as a strongly dominant mutation.

## Discussion

In this study, we show that four different mutations identified in patients with dominant myotonia shift gating of ClC-1 toward positive voltages. When coexpressed with WT subunits as in heterozygous patients, these mutants partially impose their altered voltage dependence on the heteromeric mutant/WT channel. The resulting channels are open only in a voltage range where they cannot contribute significantly to the repolarization of action potentials. This fully explains why these mutations cause myotonia and accounts for the dominant pattern of inheritance.

How general is this mechanism? Six different mutations causing dominant myotonia have been described (G230E, I290M, R317Q, P480L, Q552R, and R894X; George et al., 1993, 1994; Steinmeyer et al., 1994; Lehmann-Horn et al., 1995; Meyer-Kleine et al., 1995). However, two of these (G230E and R894X) were also found in recessive pedigrees. Coexpression studies in oocytes showed that they elicited only a weak dominant negative effect (Steinmeyer et al., 1994; Meyer-Kleine et al., 1995), and the chloride conductance predicted for heterozygotes would be at the borderline where myotonia begins. If we exclude these intermediate mutations, all known fully dominant ClC-1 mutations operate by shifting the voltage dependence of gating.

Several mutations at different positions within the channel had essentially the same effect. We conclude that not all of these mutations directly affect the "gate," but may rather affect the relative thermodynamic stabilities of the closed and open conformations. In contrast to ClC-0 (Miller, 1982; Pusch et al., 1995), at least two exponential functions are needed to fit gating kinetics of ClC-1 (G. Rychkov et al., unpublished data). This implies the existence of more than one closed or more than one open state. It is easily conceivable that changes in the conformational stabilities of these states can be achieved by mutations at very different positions.

Similar to P480L, Q552R resulted in a large shift of voltage dependence when coexpressed with WT channels, but could not be expressed by itself. By extrapolating this shift, we suggest that activation of both mutant homomers occurs at voltages where it cannot be observed experimentally. In contrast to the other mutations, activation of Q552R was also significantly slowed. Q552R has been identified in a dominant myotonic pedigree with a clinically benign form of myotonia (Lehmann-Horn et al., 1995). However, our in vitro analysis rather suggests a strong phenotype. Thus, extragenic factors may come into play.

We propose that E291K causes recessive myotonia because it leads to a misfolding of the protein. This could result in an early degradation or in an inability to associate with WT subunits (which is a prerequisite for being dominant negative). E291 is the first charged amino acid after putative transmembrane domain D5 and may define its position relative to the lipid/aqueous interface. Interestingly, the conservative mutation E291D produced an effect similar to the dominant mutations at position 290, suggesting that this region determines the stability of conformational states in a sensitive way.

In general, dominant myotonia is clinically less severe than recessive myotonia (Becker, 1977). In heterozygous patients, a proportion of WT subunits will escape inactivation by the dominant negative ones, resulting in some re-

sidual chloride conductance. Moreover, with some mutants (e.g., G230E; Steinmeyer et al., 1994), there may be partially functional WT/mutant heteromers at certain stoichiometries of assembly. Our present experiments offer an additional explanation: the changed voltage dependence of heteromeric channels could allow for a slow activation during myotonic runs, which will make these episodes shorter than with a total loss of chloride channel function. This incremental activation with fast trains of action potentials was even more pronounced with WT ClC-1 and may play a beneficial role in limiting cramps and spasms in normal musculature.

## Experimental Procedures

### Mutation Analysis in Myotonic Families

Two families of German origin with autosomal dominant (pedigree 3001) and autosomal recessive (pedigree 4012) human myotonia were investigated. For both pedigrees, linkage to the CLCN1 gene on chromosome 7q35 was shown previously (Koch et al., 1992, 1993). All affected and unaffected individuals had a neurological examination, and the phenotype was consistent with autosomal dominant myotonia congenita and recessive generalized myotonia, respectively. Venous blood samples from the family members and control probands were obtained with the approval of the ethics committee of the University of Marburg. Genomic DNA was prepared from peripheric blood leukocytes by a modification of the salting out procedure. Single stranded conformation analyses (SSCA) were performed using primers for exon 8 and 23 as published in Lorenz et al., 1994. Aberrant SSCA bands were checked for segregation in the family and for absence in at least 200 control chromosomes. These bands were then amplified and analyzed by direct sequencing of both strands of the polymerase chain reaction (PCR) product as described (Meyer-Kleine et al., 1995). All sequence variants were confirmed in PCR products derived from genomic DNA of affected family members.

### Site Directed Mutagenesis

Point mutations were introduced into the human ClC-1 cDNA (Steinmeyer et al., 1994) by recombinant PCR using Pfu DNA-polymerase (Stratagene). Briefly, two fragments were amplified with primers containing the desired mutation in a short overlapping region, joined by recombinant PCR, digested with appropriate restriction endonucleases, and ligated into the cDNA. PCR-derived fragments were entirely sequenced. The cDNAs were inserted into the vector PTLN (C. Lorenz and T. J. J., unpublished data), which contains Xenopus β-globin sequences, to boost expression (Krieg and Melton, 1984).

### cRNA Synthesis and Electrophysiology

Capped cRNA was transcribed by SP6 RNA polymerase from 0.5 μg plasmid after linearization with MluI or SnaBI using the mMessage mMachine cRNA synthesis kit (Ambion) according to the instructions of the manufacturer. 2–10 ng cRNA (corresponding to 40–200 ng/μl) was injected into Xenopus oocytes prepared and handled as described (Jentsch et al., 1990). Oocytes were kept in modified Barth's saline (88 mM NaCl, 2.4 mM $NaHCO_3$, 1.0 mM KCl, 0.41 mM $CaCl_2$, 0.33 mM $CaNO_3$, 0.82 mM $MgSO_4$, 10 mM HEPES, pH 7.6) and analyzed in ND96 saline (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4). Standard two-electrode voltage-clamp measurements were performed two days after injection at room temperature (20°C–22°C) using a Turbotec amplifier (Npi Instruments) and pCLAMP 5.5 software (Axon Instruments). With ClC-1, single channel analysis is not feasible, because of its low single-channel conductance of about 1 pS (Pusch et al. 1994).

### Data Analysis

Apparent open probability ($p_{open}$) was obtained from experiments as shown in Figure 3. After stepping the voltage to various test values (usually from +100 mV to −140 mV in 20 mV steps) for 300 ms, channel activation was monitored at a constant "tail" voltage (usually −80 mV). Extrapolated peak currents at this voltage were fitted using a Boltzmann distribution of the form:

$$I(V) = I_o + (I_{max} - I_o)/\{1 + \exp(zF[V_{½} - V]/RT)\},$$

where $I_{max}$ is the (extrapolated) current at maximal stimulation, z is the apparent gating charge, $V_{½}$ is the voltage of half-maximal activation, and $I_o$ (which was usually very small compared to $I_{max}$) is a constant offset accounting for "leak" currents endogenous to the oocyte. Apparent $p_{open}$ was obtained by the normalization $p_{open} = I(V)/I_{max}$ (cf. Figure 3B).

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**Note Added in Proof**

The data referred to as Steinmeyer and Jentsch, unpublished data, are now in press: Steinmeyer, K., Schwappach, B., Vandewalle, A., Bens, M., and Jentsch, T. J. (1995). J. Biol. Chem., in press.