

A missense mutation in canine ClC-1 causes recessive myotonia congenita in the dog¹

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Abstract Myotonia congenita is an inherited disorder of sarcolemmal excitation leading to delayed relaxation of skeletal muscle following contractions. Mutations in a skeletal muscle voltage-dependent chloride channel, ClC-1, have been identified as the molecular genetic basis for the syndrome in humans, and in two well characterized animal models of the disease: the myotonic goat, and the arrested development of righting (*adr*) mouse. We now report the molecular genetic and electrophysiological characterization of a canine ClC-1 mutation that causes autosomal recessive myotonia congenita in miniature Schnauzers. The mutation results in replacement of a threonine residue in the D5 transmembrane segment with methionine. Functional characterization of the mutation introduced into a recombinant ClC-1 and heterologously expressed in a cultured mammalian cell line demonstrates a profound effect on the voltage-dependence of activation such that mutant channels have a greatly reduced open probability at voltages near the resting membrane potential of skeletal muscle. The degree of this dysfunction is greatly diminished when heterodimeric channels containing a wild-type and mutant subunit are expressed together as a covalent concatemer strongly supporting the observed recessive inheritance in affected dog pedigrees. Genetic and electrophysiological characterization of the myotonic dog provides a new and potentially valuable animal model of an inherited skeletal muscle disease that has advantages over existing models of myotonia congenita.

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Key words: Myotonia; Chloride channel; Skeletal muscle; Animal model

1. Introduction

Myotonia congenita is an inherited condition characterized by delayed relaxation of skeletal muscle after voluntary contraction without associated symptoms of weakness or muscular dystrophy [1]. The disease may be transmitted as either an autosomal dominant (Thomsen's disease) or recessive (recessive generalized myotonia) trait. Recent molecular genetic studies have identified the *CLCN1* gene encoding the skeletal muscle voltage-dependent chloride channel, ClC-1, as responsible for this condition in human, mouse, and goat forms of the disease [2–5].

Appropriate animal models of inherited human diseases are important for understanding pathophysiology and for testing new therapies. Presently, there are two well characterized animal models of myotonia congenita. The myotonic goat provided the first opportunity to define the cellular defect in myotonic muscle. Seminal work by Bryant and colleagues, first demonstrated that muscle fibers from myotonic goats exhibit greatly diminished chloride conductance, and that this leads directly to hyperexcitability and a characteristic after-depolarization associated with elevations in transverse tubular potassium concentration [6,7]. The molecular defect in the myotonic goat, a missense mutation in the carboxy terminus of ClC-1 described by Beck et al., reduces channel open probability at physiologic voltages by altering the voltage-dependence of channel activation [5]. Transmission of the myotonic phenotype in the goat is autosomal dominant. The only other characterized animal model of myotonia congenita is the *adr* (arrested development of righting) mouse [8]. In this animal, autosomal recessive transmission of a severe myotonic phenotype has been linked to the disruption of the murine ClC-1 gene by insertion of a transposon element [4]. Other myotonic mouse variants also exist due to non-sense or missense mutations in the same chloride channel gene [9]. Both animal models have significant disadvantages for studying the physiologic impact of new therapies. Because of its size, certain experimental manipulations, such as performing electromyography, are tedious in the *adr* mouse. Furthermore, the myotonic mouse exhibits a high mortality rate in the early postnatal period. The myotonic goat is also difficult to manipulate and maintain. Other animal models more suitable to testing new therapeutic maneuvers are clearly important to identify.

Congenital myotonia has been observed in certain dog breeds, and Vite et al. recently described this phenotype in miniature Schnauzers [10]. Myotonia in this dog breed exhibits many of the same features as human myotonia congenita including moderate to severe action myotonia, muscle hypertrophy, decreased myotonia severity with continued activity ('warm-up'), and low sarcolemmal chloride conductance.

In this report we delineate a missense mutation in canine ClC-1 that is associated with autosomal recessive transmission of the phenotype in myotonic dogs. The mutation is located near recently identified pore forming segments in ClC-1 [11] and causes a profound shift in the voltage-dependence of activation. This altered voltage-dependence of activation substantially decreases channel open probability at physiologic voltages and fully explains the reduced macroscopic chloride conductance responsible for the observed sarcolemmal hyperexcitability. These findings provide molecular genetic charac-

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terization of a new and potentially valuable animal model for studying an inherited human muscle disease.

2. Materials and methods

2.1. Isolation of RNA

Skeletal muscle was obtained from a phenotypically and electromyographically normal pure-bred miniature Schnauzer (wild-type, WT) and from a myotonic miniature Schnauzer. Total RNA was isolated with Trizol (Life Technologies, Gaithersburg, MD, USA) according to the supplier's instructions.

2.2. Northern blot analysis

Total RNA (20 µg) from WT and myotonic muscle was size-fractionated on denaturing 1% agarose/1×MOPS-EDTA/6% (vol/vol) formaldehyde gels and transferred to a nylon membrane (Hybond-N, Amersham). The blot was probed with a [³²P]dCTP-labeled antisense RNA probe transcribed from human CIC-1 cDNA (nt 1–1419) using SP6 RNA polymerase. Hybridization was performed at 50°C for 20 h in 50% formamide/5×SSPE (1×SSPE is 0.18 M NaCl, 10 mM Na₂HPO₄, 1 mM EDTA)/1% SDS/0.2% Ficoll/0.2% polyvinylpyrrolidone/2 mM sodium pyrophosphate/0.3 mg/ml denatured salmon sperm DNA with a ³²P-labeled hCIC-1 RNA probe at 2×10⁶ cpm/ml, followed by washes in 0.1×SSPE/0.1% SDS at 60°C.

2.3. Isolation and cloning of canine CIC-1 cDNA

A commercially available dog skeletal muscle cDNA library constructed in λZapII (Stratagene) was screened using standard methods [12]. Duplicate plaque lifts were hybridized at 42°C for 16 h in 50% formamide/5×SSPE/4×Denhardt's solution/1% SDS/0.3 mg/ml denatured salmon sperm DNA with ³²P-labeled hCIC-1 cDNA (*NotI*-*EcoRV* fragment, nt 1–796) at 0.6×10⁶ cpm/ml. Positive clones were plaque purified, in vivo excised as pBluescript phagemids, and sequenced by dideoxynucleotide-mediated chain-termination using fluorescent dye-terminator chemistry (Perkin-Elmer/Applied Biosystems).

2.4. Single-strand conformational analysis

Total RNA (3 µg) was randomly primed for first-strand cDNA synthesis. Subsequently, double-stranded cDNAs were amplified with each of the primer sets described in Table 1.

Amplified products were denatured, electrophoresed on 0.5 MDE gels at 3 W for 14.5 h at 25°C, and visualized by silver staining. Abnormal conformers were excised, reconstituted in water for reamplification, and sequenced with both the respective forward and reverse primers using fluorescent dye-terminator chemistry.

2.5. Allele-specific oligonucleotide hybridization

Whole blood samples were obtained from 45 normal mixed breed dogs. Genomic DNA was extracted using QIAamp spin columns (Qiagen) and used in PCR amplifications containing the following oligonucleotides: 747F, 5'-GCGGTCTCAGCAAGTTT-3' and 818R, 5'-AACAACAGCCAACCTCCTA-3'. The amplified products were denatured and transferred to a nylon membrane (Hybond-N, Amersham) utilizing a slot-blot apparatus (Minifold II, Schleicher and Schuell). The blot was hybridized for 1 h at 46°C in 5×SSPE/

4×Denhardt's solution/1% SDS with a ³²P-labeled mutant oligonucleotide (793FM: 5'-GACATGCTGATGGTG-3') at 0.6×10⁶ cpm/ml. After autoradiographic detection of the mutant allele, the blot was stripped then rehybridized with an oligonucleotide matching the WT sequence (793FWT: 5'-GACATGCTGACGGTG-3').

2.6. Site-directed mutagenesis and construction of heterodimers

Site-specific mutagenesis of hCIC-1, concatemeric hCIC-1 construction, and transient transfection of tsA201 cells were performed as previously described [13]. Briefly, the mutation T268M was constructed in hCIC-1 using a single-step polymerase chain reaction mutagenesis strategy. Primers were designed to create the desired mutation and incorporate natural restriction sites for *ApoI* (nt. 742) and *BstEII* (nt. 873) in the final product. Amplifications (20 cycles) were performed using 20 ng of hCIC-1 cDNA as template and *Taq* DNA polymerase. Final products were purified by spin-column chromatography (Qiagen), digested with *ApoI* and *BstEII*, and the resulting 131 bp fragment ligated into the corresponding sites in the plasmid pRc/CMV-hCIC-1. The amplified region was sequenced entirely in the final construct to verify the mutation and to exclude polymerase errors. A tandem construct (WT-T268M) containing both WT-hCIC-1 and T268M in the same open reading frame was constructed by the approach previously described [13].

2.7. Electrophysiological recording

WT and mutant hCIC-1 channels were either stably expressed in HEK-293 cells [14] or transiently expressed in tsA201 cells (HEK-293 cells stably transformed with the SV40 large T antigen) using calcium phosphate transfection [15]. Current recordings were made using the whole-cell or the excised inside-out configuration of the patch clamp technique as previously described [16]. The bath solution contained (in mM): 140 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, and 5 HEPES. The pipette solution contained (in mM): 130 NaCl, 2 MgCl₂, 5 EGTA, 10 HEPES. All solutions were adjusted to pH 7.4 with NaOH.

To construct activation curves, the instantaneous current amplitude divided by its maximum value at a fixed test potential measured after 1.4 s prepulses to different voltages (V) was plotted vs. the preceding potential as described previously [14]. This plot yields the voltage-dependence of the relative open probability, P_{open} . The activation curves obtained in this manner were fit with a single Boltzmann and a voltage-independent value: $I(V) = \text{Amp}[1 + \exp((V - V_{0.5})/k_V)]^{-1} + \text{constant}$.

Instantaneous whole-cell currents were determined after a 200 ms prepulse to +75 mV. Using these data, the voltage-dependence of the instantaneous whole-cell chloride conductance (g), a value proportional to the single channel conductance, was calculated from $g(V) = [I(V + 5 \text{ mV}) - I(V - 5 \text{ mV})]/10 \text{ mV}$. The equation $g(V) = g_{\text{max}}/[1 + \exp((V - V_{0.5})/k_V)]$ was fit to the experimental data [14].

3. Results and discussion

To verify the presence of an mRNA transcript encoding canine CIC-1, and to exclude large scale gene disruptions as a cause for myotonia, we performed Northern blot analysis of total RNA from myotonic and wild-type (WT) dogs using a

Table 1
Oligonucleotide primer pairs used in canine CIC-1 mutation screening

Set	Forward primer	Reverse primer
1	88F: 5'-TTCAACACAGGCTCCACAGG-3'	227R: 5'-ATCCTCATCTTGCTGTCCA-3'
2	172F: 5'-TCAGACAAGGAGCAGGACAC-3'	319R: 5'-CCCAGGAGCACCAGAAAGAT-3'
3	290F: 5'-TGGTGAGAAGAAAGTTAGGA-3'	459R: 5'-TGAGAGTTAGCGGGAAGGTG-3'
4	416F: 5'-ACCAGATGCAGCCCAACCTG-3'	598R: 5'-GGCCACAACCTTGCCACAAA-3'
5	537F: 5'-CCCTGAAATGAAGACAATAC-3'	717R: 5'-TCATAGACCCACAGACAT-3'
6	627F: 5'-GGGCTGGGCGAGTGGCATT-3'	847R: 5'-TTCCGCACAGCAAAGTATG-3'
7	811F: 5'-TTGGAGGAGTGTGTTTCAGC-3'	1020R: 5'-CCACAGCAAATCCCGATGAT-3'
8	915F: 5'-GTGCTGGCCGTGTGGAACA-3'	1108R: 5'-CTTCGCCAGAAATGGCTGA-3'
9	1044F: 5'-CTGGGAGCCGTGTTTGTTGA-3'	1232R: 5'-TGTCGAATAGGGTGCTGATG-3'
10	1211F: 5'-AGAGTTGATGCTCGTGAAG-3'	1439R: 5'-TCCTACCAGCCTCCCAATG-3'
11	1412F: 5'-TCATGCTGTGTTTGTGCTA-3'	1564R: 5'-GAGACTGTGTGGGATAGTGC-3'
12	1522F: 5'-CCTGGAGGCTATGCGGTAAT-3'	1702R: 5'-AGCTTCTTGACCTGGATGAT-3'
13	1680F: 5'-GCAGCCCTCCCTCTATGAC-3'	1822R: 5'-TTCGCAATCCCCGTATGT-3'

Myotonic Dog	DML M VGCAVGVGCCFG - 267
Rat ClC-1	DIL T VGCAVGVGCCFG - 280
Torpedo ClC-0	DIL T VGCALGISCCFG - 215
Rat ClC-2	EML A AACAVGVGCCFA - 259
Rat ClC-3	EVL S AASAAGVSVAFG - 269
Yeast GEF1	EYL T AASGAGVAVAFG - 275
Plant ClC-Nt1	DLI T CGAAAGVAAAFR - 268

Fig. 1. Comparison of ClC-1 amino acid sequence in WT and myotonic dogs. Amino acid alignment of several ClC isoforms in the region containing the myotonic dog mutation (GenBank accession numbers for aligned sequences: rat ClC-1, X62894; Torpedo, ClC-0, X56758; rat ClC-2, X64139; rat ClC-3, D17521; yeast GEF1, Z23117; ClC-Nt1, X95576). The amino acid sequence of the dog ClC-1 is a partial sequence and therefore the residue numbering is not co-linear with rat ClC-1.

radiolabeled cDNA probe derived from human ClC-1 (hClC-1, nucleotides 1–1419). Under conditions of moderate stringency we observed a –4.0 kb specific hybridization signal of approximately equal intensity, in both WT and myotonic dog muscle RNA (data not shown). These data suggest that a major disruption of the canine ClC-1 gene was not the likely cause of myotonia in this animal.

We next isolated a single 1.9 kb cDNA clone from a dog muscle cDNA library and determined its sequence using a primer walking strategy. The complete nucleotide sequence of the clone revealed a high degree of nucleotide sequence identity with rat and human ClC-1 (87% human, 83% rat), and significantly less sequence identity with other known ClC isoforms (60% rat ClC-2, 33% rat ClC-3). The nucleotide sequence of the dog clone corresponds to regions of hClC-1 encoding all predicted transmembrane domains and recently identified pore forming segments.

Using the information obtained from sequencing the partial length dog ClC-1 cDNA, we designed a panel of PCR primer

pairs (Table 1) for use in mutation screening using single-strand conformational analysis [17]. Total RNA samples from both myotonic and WT dog muscle were subjected to reverse transcription-PCR using each primer pair, and the resulting set of overlapping products were electrophoresed in non-denaturing MDE gels overnight. Gel migration patterns were resolved by silver staining and were inspected for aberrant conformers. Products produced by primer pair 6 exhibited electrophoretic differences between myotonic and WT dog. The distinct conformers from WT and myotonic dog samples were isolated, reamplified, and the products purified for sequence analysis. Sequencing revealed a C to T transition predicting the replacement of a threonine residue (ACG codon) by methionine (ATG codon) in the myotonic dog ClC-1. Based on sequence alignments, this threonine residue corresponds to threonine-268 in hClC-1 and is predicted to lie within the amino-terminal region of the D5 transmembrane segment. Threonine, serine, or alanine occupies this respective position in most known ClC isoforms (Fig. 1), and therefore we considered this a candidate mutation. The nucleotide transition occurs in the context of a mutation prone CpG dimer [18].

If the threonine to methionine substitution (designated as T268M based on the hClC-1 amino acid numbering scheme) is indeed the disease-producing mutation, then we would expect it to cosegregate with the disease phenotype in affected dog pedigrees. Unfortunately, the parents of the myotonic dog from whom the muscle tissue was obtained could not be identified, and we therefore ascertained additional myotonic animals from a breeder. Breeding studies of other myotonic dogs indicate an autosomal recessive mode of inheritance [21], and we used an allele-specific hybridization assay using PCR amplified genomic DNA to perform genotype analysis. Four myotonic dogs from one three generation pedigree were ho-

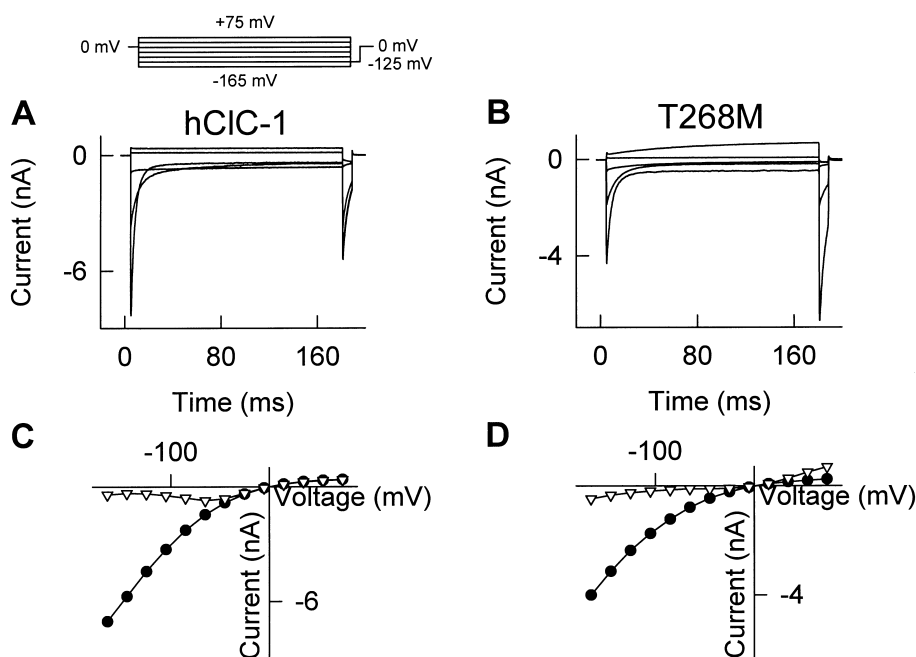


Fig. 2. Electrophysiological characterization of WT-hClC-1 and T268M. Whole-cell current recordings from tsA201 cells transfected with either WT-hClC-1 (A) or T268M (B). Currents were recorded in response to a series of test potentials ranging from –165 to +75 mV in 40 mV steps from a holding potential of 0 mV (pulse protocol illustrated above panel A). Representative current-voltage relationships for WT-hClC-1 (C) and T268M (D) are shown for both ‘instantaneous’ (●) and steady-state (▽) current amplitudes (‘instantaneous’ current amplitudes are defined as the current recorded 400 μs after the onset of the test potential).

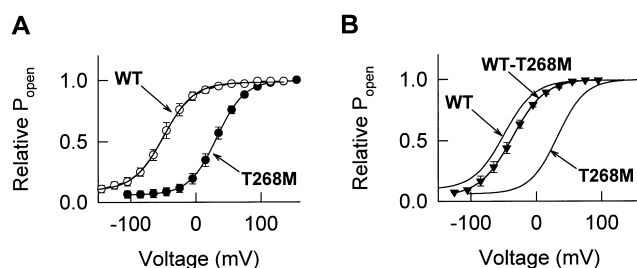


Fig. 3. Voltage-dependence of activation for WT, T268M, and WT-T268M channels. A: Comparison of WT-hClC-1 (○) and T268M (●) current amplitudes recorded after a 1.4 s prepulse to different voltages as described in the text. The solid lines represent fitted single Boltzmann functions ($n=4$ cells for each construct). B: Voltage-dependence of activation for WT-T268M heterodimer (▼) shown in comparison to fitted lines from panel A for WT and T268M homodimers ($n=4$ cells for WT-T268M). Error bars are S.E.M.

mozygous for the mutation, and two non-myotonic obligate carriers were found to be heterozygous for the allele. The allele was not present in 45 unrelated non-myotonic dogs of various breeds. The presence of a nucleotide substitution affecting a conserved amino acid, its consistent presence in all myotonic dogs ascertained and its absence in normal animals, strongly support that this is the disease producing mutation.

To further demonstrate that the mutation critically affects the function of the muscle chloride channel consistent with myotonia, we performed functional studies using the recombinant hClC-1 cDNA heterologously expressed in a cultured mammalian cell line (tsA201). We introduced the mutation, T268M, into hClC-1, transfected this into tsA201 cells, and used whole-cell patch clamp recording to characterize its functional properties. Fig. 2 illustrates a comparison of whole-cell current records and current-voltage relationships for WT and mutant hClC-1. The mutant exhibits an altered voltage-dependence of channel gating manifest by slight changes in the time course of deactivation at strongly negative test potentials (Fig. 2B), changed voltage-dependence of the late current amplitude (Fig. 2D), and a time-dependent increase of the current amplitude upon voltage steps to positive potentials (Fig. 2B). Pore properties were only minimally affected by the T268M mutation. Ion selectivity determined from permeability ratios measured under biionic conditions [15] was altered slightly in the mutant (WT-hClC-1: $\text{Cl} > \text{SCN} > \text{Br} > \text{NO}_3 > \text{I}$; vs. T268M: $\text{SCN} > \text{Cl} > \text{Br} > \text{NO}_3 > \text{I}$). Furthermore, the voltage-dependence of the unitary conductance inferred from the instantaneous conductance-voltage relationship was unchanged (data not shown).

The voltage-dependence of the relative channel open probability was tested by recording instantaneous current amplitudes following a 1.4 s prepulse to various voltages, and plotting the recorded normalized current as the relative open probability at the end of the prepulse (Fig. 3). For WT and T268M, these curves can be fit with a single Boltzmann plus a voltage-independent value denoting the minimum open probability at very negative potentials. Neither the slope factors (WT: 23.1 ± 0.6 mV, $n=4$; T268M: 21.1 ± 0.3 mV, $n=4$) nor the voltage-independent minimum values (WT: 0.09 ± 0.01 , $n=4$; T268M: 0.06 ± 0.01 , $n=4$) were different between WT and T268M. By contrast, the midpoint of activation of T268M exhibits a 81 mV shift toward the positive potential range in comparison to WT (T268M = 32.1 ± 0.4 mV vs. WT = -49.5 ± 0.7 mV, $n=4$) (Fig. 3A). This dramatic shift

results in a diminished open probability at voltages near the resting membrane potential of mammalian skeletal muscle. In a physiologic context this biophysical defect will result in a greatly diminished sarcolemmal chloride conductance in the physiologic voltage range. A similar phenomenon has been described for mutations associated with recessive [19], and dominant myotonia congenita, including the myotonic goat mutation [5,20].

The skeletal muscle chloride channel is a dimeric protein [13], and we expect that a significant fraction of ClC-1 assembly in heterozygous animals will result in heterodimers of WT and mutant subunits. To determine the functional characteristics of heterodimers and to discern whether the mutation is functionally dominant or recessive, we constructed and expressed a concatamer of WT and T268M (WT-T268M) in a single open reading frame. The tandem dimer gave rise to functional chloride channels in tsA201 cells that strongly resemble those recorded from WT-hClC-1 (data not shown). Despite the dramatic effect of homodimeric T268M on the voltage-dependence of activation, WT-T268M exhibits a much smaller 13 mV shift in the midpoint of activation (WT-T268M = -36.9 ± 0.5 mV, $n=4$, Fig. 3B). This finding suggests that the mutant does not exhibit a dominant negative influence over the wild-type allele, and is consistent with recessive inheritance.

Myotonia congenita is one of numerous inherited diseases of skeletal muscle that have been defined at the molecular level in recent years. Although the disorder is rare and is associated with only mild morbidity, it represents an extremely well characterized pathophysiological entity that has provided a great deal of information regarding normal muscle excitation and contraction. Newer drugs and possibly novel genetic techniques require vigorous animal testing prior to their deployment on human subjects. We have defined the molecular genetic basis for autosomal recessive myotonia congenita in a breed of miniature Schnauzers. Myotonia in this animal is caused by a missense mutation in the canine ClC-1 chloride channel that causes a dramatic shift in the voltage-dependence of its open probability. These findings establish the myotonic dog as a potentially valuable new animal model of this disease process, and we believe that it will be superior to the existing models of myotonia congenita.

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