HHS Public Access

Author manuscript

Ann Neurol. Author manuscript; available in PMC 2021 August 01.

Published in final edited form as:

Ann Neurol. 2020 August ; 88(2): 297–308. doi:10.1002/ana.25780.

TRPV4 antagonism prevents mechanically-induced myotonia

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Abstract

Objective—Myotonia is caused by involuntary firing of skeletal muscle action potentials and causes debilitating stiffness. Current treatments are insufficiently efficacious and associated with side effects. Myotonia can be triggered by voluntary movement (electrically-induced myotonia) or percussion (mechanically-induced myotonia). Whether distinct molecular mechanisms underlie these triggers is unknown. Our goal was to identify ion channels involved in mechanically-induced myotonia and to evaluate block of the channels involved as a novel approach to therapy.

Methods—We developed a novel system to enable study of mechanically-induced myotonia using both genetic and pharmacologic mouse models of myotonia congenita. We extended *ex vivo* studies of excitability to *in vivo* studies of muscle stiffness.

Results—As previous work suggests activation of transient receptor potential vanilloid 4 (TRPV4) channels by mechanical stimuli in muscle, we examined the role of this cation channel. Mechanically-induced myotonia was markedly suppressed in TRPV4-null muscles and in muscles treated with TRPV4 small molecule antagonists. The suppression of mechanically-induced myotonia occurred without altering intrinsic muscle excitability such that myotonia triggered by firing of action potentials (electrically-induced myotonia) was unaffected. When injected intraperitoneally, TRPV4 antagonists lessened the severity of myotonia *in vivo* by approximately 80%.

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Author contributions: MR, CS and AV contributed to the conception and design of the study. CD, KN, KD, JM, JS, PW, NB, DL, LB and CL contributed to acquisition and analysis of data. CD, KN, KD, JM and JS contributed to preparing the figures. JS, AV, CS and MR drafted the manuscript.

Potential conflicts of interest: Nothing to report.

Interpretation—These data demonstrate for the first time that there are distinct molecular mechanisms triggering electrically- and mechanically-induced myotonia. Our data indicates that activation of TRPV4 during muscle contraction plays an important role in triggering myotonia *in vivo*. Elimination of mechanically-induced myotonia by TRPV4 inhibition offers a new approach to treating myotonia.

Introduction

Muscle stiffness due to involuntary contraction of muscle (myotonia) is present in a number of dystrophic and non-dystrophic muscle diseases [1–4]. The inability of muscle to relax rapidly following cessation of voluntary contraction leads to motor disability, which adversely affects quality of life [2, 4, 5]. The mainstay of therapy for myotonia is drugs that block Na^+ channels, such as mexiletine [2, 5–8]. Block of Na^+ channels reduces muscle excitability such that it is harder to trigger action potentials. While this may reduce myotonia, it can also lead to weakness due to impaired action potential generation during voluntary movement [9]. In addition, block of Na^+ channels can alter cardiac excitability increasing the risk of arrhythmias. Many patients suffer side effects or incomplete symptom resolution, highlighting the need for novel treatments [2, 5–8].

Myotonia can be triggered in two ways: by previous firing of voluntary action potentials or by mechanical stimulation of muscle. Myotonia triggered by voluntary action potentials we term electrically-induced myotonia. Contributors to the generation of electrically-induced myotonia include build-up of K^+ in the transverse tubules (t-tubules) and activation of a Na^+ persistent inward current (NaPIC) [10–13]. Mechanically-induced myotonia can be elicited by tapping muscle with a reflex hammer (percussion myotonia) [1, 2]. The contribution of mechanically-induced myotonia to motor dysfunction has never been determined.

To our knowledge, mechanisms underlying mechanically-induced myotonia have not been previously examined. One potential mechanism is the activation of mechanosensitive ion channels. A number of mechanosensitive channels have recently been described and they play central roles in diverse processes such as the sensation of touch, hearing and regulation of blood pressure [14]. Skeletal muscle has been shown to express several mechanosensitive channels [15, 16].

TRPV4 is a member of the transient receptor potential (TRP) ion channel family that is activated by mechanical stimuli, heat, hypo-osmolarity and arachidonic acid metabolites [17, 18]. In urothelial cells, TRPV4 mediates mechanically-evoked elevations of intracellular Ca^{2+} and plays a central role in regulation of bladder function [19, 20]. TRPV4 is expressed in skeletal muscle [21–23] and has been implicated in modulation of resting Ca^{2+} influx and muscle fatigue [24, 25]. Mechanically-activated currents present in wild type skeletal muscle are absent in TRPV4-null mice, strongly suggesting that TRPV4 contributes to mechanically-activated currents in skeletal muscle [26].

TRPV4 is most permeant to Ca^{2+} [27] such that its activation would be expected to depolarize muscle. Combining this with the finding that TRPV4 is activated following mechanical stimuli led us to explore the possibility that activation of TRPV4 triggers mechanically-

induced myotonia. Our data demonstrate that mechanically-induced activation of TRPV4 is a significant contributor to triggering of myotonia *in vivo* such that TRPV4 antagonism may offer a new approach to treatment of myotonia.

Methods

Mice

All animal procedures were performed in accordance with the policies of the Animal Care and Use Committee of Wright State University. The genetic mouse model of myotonia congenita used was *Clcn1*$^{adr\text{-}mto}$/J (ClC^{adr}) mice, which have a null mutation in the *Clcn1* gene (Jackson Laboratory Stock #000939). Genotyping was performed as previously described to select heterozygous mice for breeding [28]. Otherwise, homozygous myotonic mice were identified by appearance and behavior as previously described [29]. To pharmacologically induce myotonia, muscle from asymptomatic littermates was treated with 100 μM 9-anthracenecarboxylic acid (9AC) to block ClC-1 chloride channels [30]. Mice were used from 6 weeks to 4 months of age.

TRPV4-null (*Trpv4*$^{-/-}$) mice were generated by homologous recombination using a targeting vector including loxP sites flanking *Trpv4* exons 4 and 5, followed by outcrossing to Sox2^{Cre}-expressing mice (Jackson Laboratory Stock #004783; Fig 1A). Quantitative reverse transcription PCR (RT-qPCR) analyses confirm the absence of *Trpv4* mRNA expression in these mice (Fig 1B). *Trpv4*$^{-/-}$ mice and wild type littermates were generated by interbreeding of *Trpv4*$^{+/-}$ mice. Genotyping was performed by PCR analysis of tail DNA using the following primers: F1, 5′-GACAGCTGTGTCTCCACCAA; F2, 5′-GTGAGTAGCGGTGGAGGCTA; R1, 5′-GGACCAAAATGAGGGTGTGAACTTT (Fig 1A). This multiplex PCR produces a 183-bp amplicon for the wild type allele, a 200-bp amplicon for the null allele, and both amplicons in heterozygous mice. Comparable to previously reported *Trpv4* knockout mouse lines [31], *Trpv4*$^{+/-}$ mice from this newly generated line did not exhibit deficits in weight, survival, or motor function (data not shown).

Intracellular recording from individual myofibers

Mice were sacrificed using CO_2 inhalation followed by cervical dislocation, and both extensor digitorum longus muscles were dissected out tendon-to-tendon. Muscles were maintained at 20–22°C and recorded within 6 hours of sacrifice. The recording chamber was continuously perfused with Ringer solution containing (in mM): NaCl, 118; KCl, 3.5; $CaCl_2$, 1.5; $MgSO_4$, 0.7; $NaHCO_3$, 26.2; NaH_2PO_4, 1.7; glucose, 5.5 (pH 7.3–7.4), and equilibrated with 95% O_2 and 5% CO_2.

Intracellular recordings were performed as previously described [13, 29]. Briefly, muscles were loaded with 50 μM N-benzyl-p-toluenesulfonamide (BTS) for at least 30 minutes prior to recording to prevent contraction [32]. To aid in visualization of fibers during impalement, muscle was stained for 3 minutes with 10 μM 4-(4-diethylaminostyrl)-N-methylpyridinium iodide (4-Di-2-ASP) and imaged with an upright epifluorescence microscope. Micro-electrodes were filled with 3M KCl solution containing 1 mM sulforhodamine 101 to

visualize the electrodes with epifluorescence. Resistances were between 15 and 30 MΩ, and capacitance compensation was optimized prior to recording. To perform intracellular recordings of action potentials, individual fibers were impaled with two sharp electrodes, one to pass current and one to record the voltage response. For comparisons of vehicle treatment to drug treatment the bilateral EDL muscles were dissected out. One was treated with vehicle and the other with drug. This allowed for paired recordings from single mice for vehicle versus drug treatment.

Fibers with resting potentials more depolarized than –74 mV were excluded from analysis. Passive properties and properties of single action potentials were measured as previously described [28]. Action potential voltage threshold was defined as the voltage at which the dV/dt was 0.03 of its maximum (a value near 10 mV/ms) based on methods described by Sekerli et al [33]. Input resistance was calculated by injecting hyperpolarizing current and measuring the steady-state response.

Reverse transcription quantitative PCR (RT-qPCR)

Tissues were homogenized in TRIzol (Thermo Fisher) utilizing Lysing Matrix D 1.4 mm ceramic spheres (MP Biomedicals) and a FastPrep-24 5G Instrument (MP Biomedicals). Total RNA was then isolated and converted to cDNA as described previously [34]. RT-qPCR analyses were performed using an ABI 7900HT Real-Time PCR System (Thermo Fisher), with Taqman Universal PCR Master Mix (Thermo Fisher) and the following Taqman Gene Expression Assays: *Gusb*, Mm00446953_m1 (Thermo Fisher); *Trpv4*, Mm00499025_m1 (Thermo Fisher). *Trpv4* transcript levels were quantified using the delta-delta Ct (ΔΔCt) method with normalization to the reference gene *Gusb*. Each reaction was performed in triplicate and all data are presented as average normalized *Trpv4* transcript levels relative to a single wild type sample, which was assigned the value of 1.

Drugs

HC-067047 was obtained from Hello Bio (Princeton, NJ), GSK2193874 from Tocris (Minneapolis, MN), 9AC and sulforhodamine 101 from Sigma Aldrich (St. Louis, MO), BTS from TCI America (Portland, OR), and 4-Di-2-ASP from Molecular Probes (Eugene, OR).

In vivo force recording

In vivo muscle force recordings were performed as previously described [28]. Mice were anesthetized via isoflurane inhalation; then the distal tendon of the triceps surae was attached to a force transduction motor and the sciatic nerve stimulated while isometric muscle force generation was measured. Muscle temperature was monitored with a laser probe and maintained between 28 ˚C and 30 ˚C with a heat lamp. The muscle was kept moist by applying mineral oil. Force recordings in ClC^{adr} mice were performed before and 30 minutes following intraperitoneal (IP) injection. For HC-067047 (20 mg/kg), 0.2 ml of solution containing drug or vehicle was injected (0.015 ml of DMSO and 0.185 ml of saline). GSK2193874 was first dissolved in DMSO at a concentration of 7.5 mg/ml (0.375 mg/50 µl DMSO) using sonication. Distilled water was then added in a 1:1 ratio, followed

by another round of sonication. Close to 0.1 mls of this solution was injected IP, with the volume adjusted to administer the correct dose of drug.

Statistical analysis and blinding

For statistical comparisons of intracellular recordings from individual muscle fibers, a repeated measures ANOVA with a random effect for mouse was run using SAS version 9.4 (SAS Institute, Inc., Cary, NC). The “simulate” post-hoc multiple comparison procedure was used for all post-hoc multiple comparisons. Origin software (OriginLab, Northampton, MA) was used to perform paired t-tests for analysis of *in vivo* force recordings before and after administration of HC-067047 or GSK2193874. All data are presented as means ± SEM, $p < 0.05$ was considered to be significant. The numbers of mice and muscle fibers used are described in the corresponding figure legends and text. For statistical analysis “n” was the number of mice.

For *ex vivo* experiments comparing mechanically-induced myotonia in *Trpv4*$^{+/+}$ to *Trpv4*$^{-/-}$ mice, the experimenter was blinded as to the genotype of the mice. Similarly, *ex vivo* experiments testing efficacy of drugs were performed with the experimenter blinded as to which vial contained drug and which contained vehicle. For *in vivo* experiments, as the drug solution was opaque, GSK2193874 solutions were taken up in a syringe by a different experimenter and the syringe wrapped in tape to maintain blinding until analysis of data was complete.

Results

We studied muscle isolated from two mouse models of myotonia congenita: i) a pharmacologic model in which ClC-1 chloride channels are blocked with 100 μM 9-anthracenecarboxylic acid (the 9AC model) [30]; and ii) a genetic model (*Clcn1*$^{adr\text{-}mto}$/J, ClC^{adr} mice) in which affected mice are homozygous for a null mutation in the *Clcn1* gene [35]. In each model of myotonia we studied the response of skeletal muscle to both electrical stimulation and mechanical stimulation, two distinct triggers of myotonia.

Muscle isolated from wild type mice responded to a 200 ms current injection of depolarizing current by repetitively firing action potentials that immediately ceased when current injection was terminated (Fig 2A). In contrast, muscles from both models of myotonia congenita responded to current injection with firing of action potentials that persisted following cessation of current injection (Fig 2A). We define this as electrically-induced myotonia to distinguish it from mechanically-induced myotonia.

In order to study mechanically-induced myotonia, it was necessary to develop a system that allowed for intracellular recording from individual muscle fibers during mechanical displacement of the fiber. For these studies, fibers were impaled with a single, sharp recording electrode. Approximately 200 μm away from the sharp electrode, a blunt 50 μm diameter, polished glass pipette was rested against the impaled fiber. The 200 μm distance was selected to be as close as possible, yet far enough away that pushing on the fiber with the blunt electrode did not cause loss of impalement (Fig 2B). The blunt electrode was manually advanced ~20 μm while recording the myofiber’s response to mechanical

stimulation with the intracellular electrode. This distance was found to trigger mechanically-induced myotonia in all myotonic fibers. To avoid bias in manual advancement of the blunt electrode the experimenter was blinded as to genotype or drug treatments in all experiments of mechanically-induced myotonia.

In wild type muscle, mechanical stimulation triggered a depolarization of less than 3 mV that did not trigger myotonia (56 fibers from 8 mice, Fig 2C). In contrast, in myofibers from both models of myotonia congenita, 100% of myofibers exhibited mechanically-induced myotonia (Fig 2C). To directly measure the depolarization triggered by mechanical stimulation, action potentials were blocked with 1 μM tetrodotoxin (TTX; Fig 2D). In myofibers derived from the 9AC model of myotonia congenita, the depolarization averaged 8.6 ± 0.5 mV (35 fibers from 5 mice). In myofibers from the ClC^{adr} model, it averaged 9.5 ± 0.6 mV (21 fibers from 3 mice). To our knowledge, this is the first measurement of mechanically-induced depolarization of skeletal muscle.

It has been suggested that mechanically-activated currents are absent in muscle lacking TRPV4 ion channels [26]. To determine whether TRPV4 might contribute to mechanically-induced myotonia, we first assessed *Trpv4* transcript levels in the extensor digitorum longus muscle (EDL), the muscle studied physiologically here. RT-qPCR analyses indicated that *Trpv4* transcript was detectable in the EDL, with equivalent expression levels in wild type and ClC^{adr} mice (Fig 3A, 1.05 ± 0.07 wild type vs 1.19 ± 0.10 ClC^{adr}, p = 0.27).

To determine whether activation of TRPV4 plays a role in triggering mechanically-induced myotonia, we recorded from muscle from $Trpv4^{-/-}$ mice. Muscles from wild type siblings and $Trpv4^{-/-}$ mice were treated *ex vivo* with 9AC to cause myotonia and mechanically stimulated with a blunt electrode. In muscles from $Trpv4^{+/+}$ mice, mechanically-induced myotonia was present in 21/28 fibers from 4 mice (Fig 3B). In $Trpv4^{-/-}$ mice, mechanically-induced myotonia was present in 0/28 fibers from 4 mice (Fig 3A). The elimination of mechanically-induced myotonia in 9AC-treated $Trpv4^{-/-}$ muscle was not accompanied by changes in membrane properties or properties of single action potentials (Table 1). All 9AC-treated $Trpv4^{+/+}$ and $Trpv4^{-/-}$ muscle fibers had electrically-induced myotonia (Fig 3C). Following addition of TTX to block action potentials, $Trpv4^{-/-}$ fibers exhibited a close to 50% reduction in mechanically-induced depolarization relative to wild type myofibers (5.6 ± 0.5 mV in $Trpv4^{+/+}$ vs 2.8 ± 0.5 in $Trpv4^{-/-}$, $p < 0.01$, n = 28 fibers from 4 mice for each group). These data demonstrate that loss of TRPV4 selectively eliminates mechanically-induced myotonia by decreasing mechanically-induced depolarization of muscle.

To determine whether small molecule antagonism of TRPV4 might be useful as therapy for myotonia, we examined whether antagonism of TRPV4 could lessen mechanically-induced myotonia *ex vivo*. HC-067047 at a dose of 1 μM has previously been shown to fully inhibit TRPV4 in urothelial cells [20]. Muscle from ClC^{adr} mice was exposed to either 1 μM HC-067047 or DMSO vehicle at a final concentration of 0.1%. The response to mechanical stimulation was dramatically altered by application of HC-067047. After application of vehicle, 100% of fibers exhibited mechanically-induced myotonia (28/28 fibers from 4 mice, Fig 4A), whereas following application of HC-067047, no fibers exhibited mechanically-induced myotonia (0/28 fibers from 4 mice, Fig 4A). To quantitate the effect of HC-067047

on the mechanically-induced depolarization, 1 μM TTX was added to the bath to block generation of action potentials. ClC^{adr} muscle fibers depolarized by 9.5 ± 0.6 mV following mechanical stimulation. Treatment with 1 μM HC-067047 decreased this to 5.5 ± 0.6 mV ($p < 0.01$ vs vehicle, n = 21 vehicle-treated fibers from 3 mice and 21 HC-067047-treated fibers from 3 mice). Application of HC-067047 had no effect on mechanically-induced depolarization in *Trpv4*$^{-/-}$ muscle (2.8 ± 0.5 vs 2.7 ± 0.4 mV, n = 28 fibers from 4 mice for each group). These data strongly suggest that HC-067047 abolishes mechanically-induced myotonia by blocking TRPV4 channels.

Application of HC-067047 did not eliminate electrically-induced myotonia (Fig 4B). After treatment with either vehicle or HC-067047, electrically-induced myotonia was present in all fibers (33/33 vehicle-treated fibers from 5 mice and 48/48 HC-067047-treated fibers from 5 mice, Fig 4B). Similar results were obtained using 1 μM GSK2193874 to block TRPV4 channels [36]. Application of GSK2193874 greatly reduced the incidence of mechanically-induced myotonia (19/20 fibers with mechanically-induced myotonia in 3 mice treated with vehicle and 3/20 fibers with mechanically-induced myotonia in 3 mice treated GSK2193874, Fig 4C) while having no effect on the incidence of electrically-induced myotonia (21/21 fibers from 3 mice for vehicle and 19/19 fibers from 3 mice for GSK2193874, Fig 4D). These data suggest that drugs that block TRPV4 selectively antagonize mechanically-induced myotonia.

Muscle excitability is determined by the amount of depolarizing current or depolarization of membrane potential required to trigger an action potential. In hyperexcitable myotonic muscle, muscle is too easily depolarized to action potential threshold, while in hypoexcitable muscle it is difficult to reach action potential threshold. To determine whether HC-067047 and GSK2193874 were eliminating mechanically-induced myotonia via a reduction in excitability, we measured passive membrane properties and properties of single action potentials. These properties of muscle determine the difficulty in reaching action potential threshold. Resting potential, input resistance and properties of single action potentials were unchanged by either drug (Table 1), suggesting neither drug altered excitability.

To determine whether antagonism of TRPV4 might be effective as therapy, we measured the severity of myotonia using *in vivo* contractile force recordings of gastrocnemius muscle as described previously [28]. The sciatic nerve was stimulated at 60 Hz while measuring isometric muscle force generation. In wild type muscle, force fused during 60 Hz stimulation and relaxed immediately upon cessation of stimulation (Fig 5A). In ClC^{adr} muscle, fusion of force was normal during stimulation, but relaxation following termination of stimulation was only partial due to myotonia (Fig 5B). Both amplitude and duration of muscle force have been previously used to estimate severity of myotonia [37]. To combine these into a single value we took the integral of force with respect to time [9]. To eliminate differences in measurement of myotonia due to differences in muscle force between mice, the integral of myotonia was normalized to force generated by a single stimulus before and after administration of drug. We measured severity of myotonia before and 30 minutes after IP injection of 25 mg/kg GSK2193874, 20 mg/kg HC-067047 or vehicle in ClC^{adr} mice. Following injection of vehicle there was no change in severity of myotonia (Fig 5B–C). Following injection of both GSK2193874 and HC-067047 myotonia was reduced by over

80% (Fig 5B–C, $p < 0.01$ versus vehicle for both drugs, n = 5 mice for each drug treatment group and 6 for vehicle).

Discussion

Current treatment of myotonia is only partially effective and can cause unwanted side effects due, in part, to nonspecific suppression of excitability of both cardiac and skeletal muscle. Identifying currents in skeletal muscle that contribute to triggering of myotonia may lead to development of better targeted therapies that treat muscle stiffness secondary to myotonia without unwanted side effects. We established a novel *ex vivo* system to study currents responsible for mechanically-induced (percussion) myotonia. Genetic deletion of TRPV4 suppressed generation of mechanically-induced myotonia, while having no effect on myotonia triggered by previous firing of action potentials (electrically-induced myotonia). These data provide the first definitive evidence of distinct molecular mechanisms underlying electrically- and mechanically-induced myotonia. Small molecule inhibitors of TRPV4 reduced the incidence of mechanically-induced myotonia *ex vivo* and treatment of mice with systemic administration of these inhibitors also significantly reduced myotonia triggered by repetitive stimulation of the sciatic nerve *in vivo*. Our findings suggest antagonism of TRPV4 may offer a novel therapeutic approach to treating myotonia. A particular advantage of TRPV4 blockade is that it did not affect muscle excitability (the ease of generating action potentials) such that unwanted side effects of weakness and altered cardiac excitability might be avoided.

Two molecularly distinct triggers of myotonia

It has been known for many years that myotonia can be triggered by either voluntary firing of action potentials or percussion of muscle [1, 2]. We term these electrically- and mechanically-induced myotonia, respectively. Although it has been presumed that distinct mechanisms are involved, direct proof has been lacking. One reason for the lack of progress in identifying the currents responsible for generation of mechanically-induced myotonia has been the inability to record intracellularly from intact muscle fibers during mechanical stimulation of muscle. We developed a system that allowed for intracellular recording of the depolarization of membrane potential and the subsequent myotonia triggered by mechanical stimulation of individual fibers. This made it possible to determine that genetic deletion of TRPV4 ion channels greatly reduced mechanically-induced myotonia, while having no effect on electrically-induced myotonia.

Shown in Figure 6 is a depiction of our current understanding of the distinct mechanisms underlying electrically- and mechanically-induced myotonia in the muscle disease myotonia congenita. In myotonia congenita there is a reduction in the number of functional Cl^- channels. ClC-1-mediated Cl^- conductance accounts for 70–80% of resting muscle membrane conductance and stabilizes the membrane potential near the resting potential [35, 38–40]. In the absence of Cl^- current, stimuli that would otherwise cause only modest depolarization, lead to depolarization large enough to trigger myotonia.

In electrically-induced myotonia, an important source of depolarization is exit of K^+ from the fiber during repolarization of action potentials. The exit of K^+ leads to build-up of K^+ in

t-tubules, a depolarized shift in the K^+ equilibrium potential and depolarization of the membrane potential [10–12, 38]. When the Cl^- current is small or absent the depolarization caused by K^+ build-up triggers myotonia.

In mechanically-induced myotonia, there have been no preceding action potentials so build-up of K^+ in t-tubules cannot be a contributor to depolarization. Instead, mechanical distortion of the sarcolemma results in opening of TRPV4 channels, which are nonselectively permeable to monovalent and divalent cations [27], such that their activation causes depolarization and myotonia. A prediction of this model of mechanically-induced myotonia is that agonists of TRPV4 channels would worsen myotonia, though this remains to be tested experimentally.

Ex vivo experiments demonstrated that electrically- and mechanically-induced myotonia could be triggered independently. However, *in vivo*, block of TRPV4 channels significantly reduced the severity of myotonia triggered by repetitive stimulation of the sciatic nerve (electrically-induced myotonia). The difference is likely due to experimental conditions. During *ex vivo* studies muscle contraction was eliminated with BTS, a drug that blocks interaction of actin and myosin [32, 41] because contraction of muscle during intracellular recording causes damage-induced loss of membrane potential. During *in vivo* experiments, muscle contraction was not blocked so that force could be measured. Thus, firing of action potentials *in vivo* increased tension as muscle contracted, which appears to activate TRPV4 to worsen myotonia. We conclude that build-up of K^+ in t-tubules and activation of TRPV4 combine to trigger myotonia *in vivo*. The lessening of myotonia following block of TRPV4 strongly suggests mechanically-induced activation of TRPV4 is a contributor to generation of myotonia *in vivo*.

Mechanisms underlying mechanically-induced depolarization of muscle

The simplest explanation for the contribution of TRPV4 to mechanically-induced myotonia is that TRPV4 is activated by mechanical forces exerted on the sarcolemma. Several *in vitro* studies have provided evidence that TRPV4 is insensitive to membrane stretch [42–45], but can be activated by mechanical stimuli applied at cell-substrate contacts [43] or to cell surface β1-integrins [46]. Given the importance of cell-matrix interactions to skeletal muscle physiology [47], cell-substrate contacts may play a key role in sarcolemmal TRPV4 activation in response to mechanical stimulation. However, little is currently known about how TRPV4 is activated by mechanical stimuli *in vivo*. It also remains to be determined whether the biomechanical properties of skeletal muscle change in the context of myotonia and whether such changes alter the sensitivity of TRPV4 to mechanical stimuli.

In several cell types TRPV4 activation in response to osmomechanical stress occurs with a latency of seconds and is mediated by phospholipase A2 (PLA_2)-dependent generation of second messengers, such as arachidonic acid [48–51]. In contrast, TRPV4 activation by deflection at cell-substrate interfaces occurs with a rapid latency (<2 ms) and is PLA_2-independent, consistent with direct transduction of the mechanical stimulus [43]. Similarly, calcium influx through TRPV4 resulting from the application of mechanical force to β1-integrins occurs with a latency of milliseconds [46]. The rapid onset of mechanically-induced myotonia observed in both the pharmacologic and genetic models described here (Figure 2)

suggests TRPV4 activation in this context may be mediated by mechanisms comparable to those underlying channel responses to cell-substrate deflections or integrin stimulation, rather than via activation by second messengers.

It remains to be determined whether all mechanically-activated currents contributing to triggering of myotonia have been identified. While there is one study suggesting genetic deletion of TRPV4 abrogates mechanically activated currents in muscle [26], we did not observe elimination of mechanically-induced depolarization. The remaining depolarization may be due to a technical issue, such as depolarization by the impaling electrode during mechanical stimuli. Alternatively, a second mechanically activated channel expressed in muscle such as Piezo1, TRPC1, TRPC6 or TRPV2 might also contribute to generation of mechanically-induced myotonia [21, 22, 52–55].

TRPV4 antagonists as a novel approach to therapy of myotonia

Myotonia is present in muscle diseases such as myotonia congenita, myotonic dystrophy, sodium channel myotonia, paramyotonia congenita and hyperkalemic periodic paralysis [1–4]. Myotonia causes functionally limiting muscle stiffness, weakness and pain [2, 4, 5]. For movement to occur normally, muscle needs to sequentially contract and relax during flexion and extension of a joint. For this to occur, muscle must fire action potentials during contraction, and be electrically silent during relaxation as antagonist muscles contract and the muscle is stretched as it lengthens. If muscle is unable to relax while lengthening, due to mechanically-induced myotonia, this will negatively impact motor performance. The contribution of mechanically-induced myotonia to motor dysfunction in patients with myotonia has never been determined, as it has not been possible to selectively block mechanically-induced myotonia. As described above, our studies suggest mechanically-induced myotonia is a significant contributor to motor dysfunction *in vivo* such that block of mechanically-induced myotonia may improve motor performance.

The current approach to therapy of myotonia is focused on block of Na^+ channels responsible for generation of action potentials [2, 5–8]. This reduces excitability and thus reduces myotonia. A problem with this approach is that activation of Na^+ channels is required during the action potentials necessary for voluntary movement. Thus, care must be taken to not block too high a percentage of Na^+ channels, so as not to cause weakness/paralysis.

A superior approach to therapy would be to block pathologic excitation of muscle without affecting normal excitation [9]. Knockout of TRPV4 had no effect on properties of single action potentials that depend on Na^+ current, suggesting that excitability is not significantly reduced. Thus, it may be possible to reduce myotonia by blocking TRPV4 without adversely affecting strength during voluntary movement. It has been reported that there are muscle transcriptional changes in *Trpv4*$^{-/-}$ mice, and that TRPV4 activation modulates resting Ca^{2+} influx and muscle fatigue [24, 25], such that block of TRPV4 may have side effects related to muscle function. However, despite TRPV4 being expressed in a number of tissues, knockout mice have a surprisingly mild phenotype, suggesting that block of TRPV4 *in vivo* might not lead to intolerable side effects [31]. Administration of selective TRPV4 blockers is well tolerated in both rodents and patients [36, 56]. Thus, using block of TRPV4 as therapy for

patients with myotonia may become possible in the near future. One approach would be to add block of TRPV4 to current therapy, as there would be little risk of causing muscle hypoexcitability.

The studies reported here focused on myotonia resulting from loss of muscle Cl^- current. However, as TRPV4 channels are present in wild type muscle, it is possible that activation of TRPV4 contributes to muscle stiffness in other diseases with myotonia. Thus, block of TRPV4 may be useful in treating myotonia in diseases such as myotonic dystrophy, sodium channel myotonia, paramyotonia congenita and hyperkalemic periodic paralysis.

Acknowledgements

This work was supported by NIH grants AR074985 (M.M.R.), NS087579 (C.J.S) and NS099850 (A.A.V.) and MDA grant 602459 (M.M.R.).

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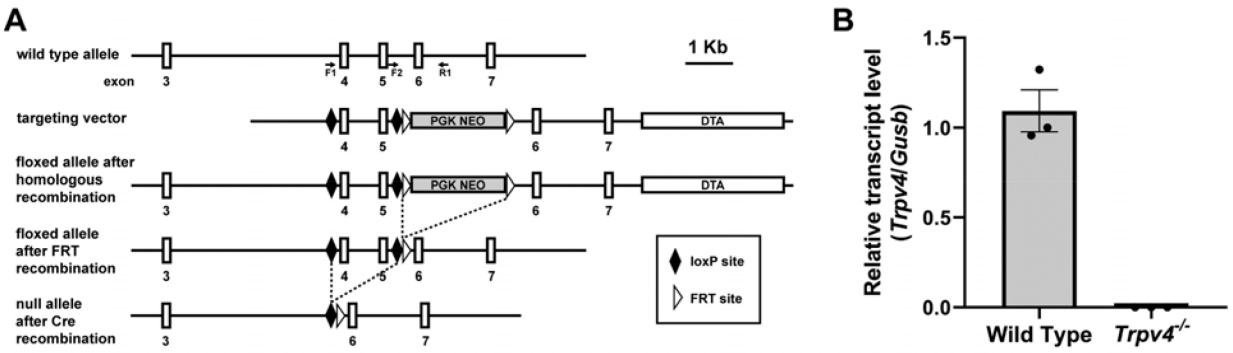

Figure 1:

Generation and characterization of *Trpv4*$^{-/-}$ mice. (A) Schematic representation of the targeting strategy. The structure of the endogenous mouse *Trpv4* allele is shown at top, including the locations of the three genotyping primers, above the targeting vector in which *Trpv4* exons 4 and 5 are flanked by loxP sites. Following excision of the Neo cassette, mice were outcrossed to a Sox2^{Cre}-expressing mouse strain enabling Cre recombinase (Cre)-mediated gene deletion. (B) *Trpv4* mRNA levels in extensor digitorum longus muscle of adult WT and *Trpv4*$^{-/-}$ mice, as assessed by RT-qPCR (n=3 for WT and *Trpv4*$^{-/-}$).

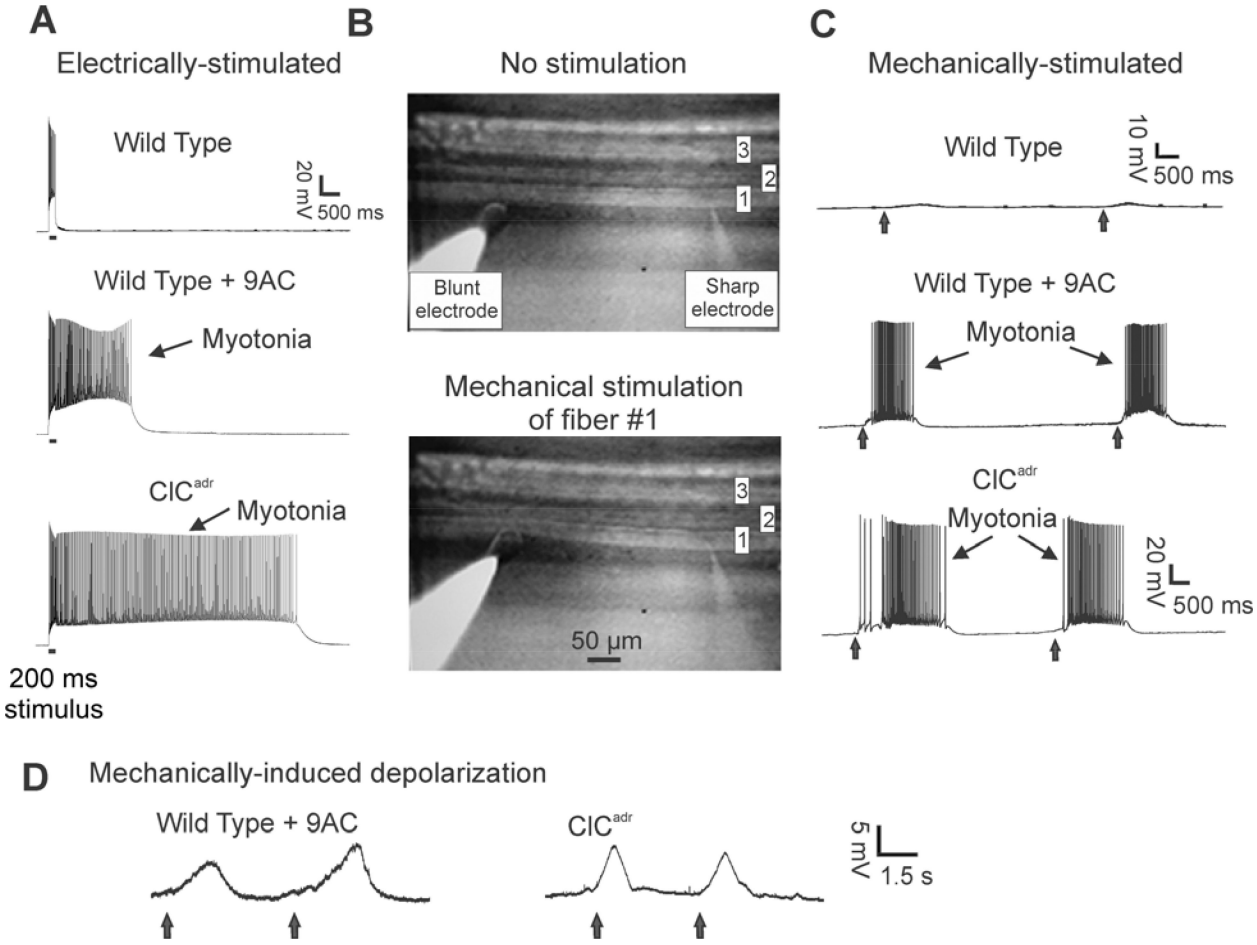

Figure 2:

Electrically- and mechanically-induced myotonia are present in both pharmacologic and genetic mouse models of myotonia congenita. A) Intracellular recording from muscle fibers during stimulation by injection of depolarizing current. In each of the three traces, a 200 ms injection of current (horizontal line under the trace) triggers repeated firing of action potentials during the current injection. In wild type muscle, there is no firing of action potentials following termination of current injection (0/63 fibers from 7 mice). In wild type muscle myofibers in which ClC-1 chloride channels are blocked with 100 µM 9AC and in muscle from ClC^{adr} mice, there is continued firing of action potentials (myotonia) following termination of current injection (58/58 fibers from 8 mice and 72/72 fibers from 9 mice, respectively). B) The arrangement of electrodes used to mechanically stimulate individual muscle fibers. Three muscles fibers are in focus in the image shown. A sharp electrode is impaled into fiber #1 on the right of the image. On the left, a blunt electrode is resting on the bottom of fiber #1, over 200 µm away from the sharp recording electrode. In the top image (No stimulation), the blunt electrode is gently resting on fiber #1. In the lower image the blunt electrode has been manually advanced to mechanically stimulate fiber #1. C) At the times indicated by vertical arrows, the blunt electrode was manually advanced. In wild type muscle, mechanical stimulation did not trigger myotonia (0/56 fibers from 8 mice). In both 9AC-treated and ClC^{adr} muscle, mechanical stimulation triggered myotonia in all fibers

tested (42/42 fibers from 6 mice and 56/56 fibers from 8 mice, respectively). D) Examples of mechanically-induced depolarization in muscle when 1 μM TTX was added to the perfusate to block Na^+ channels.

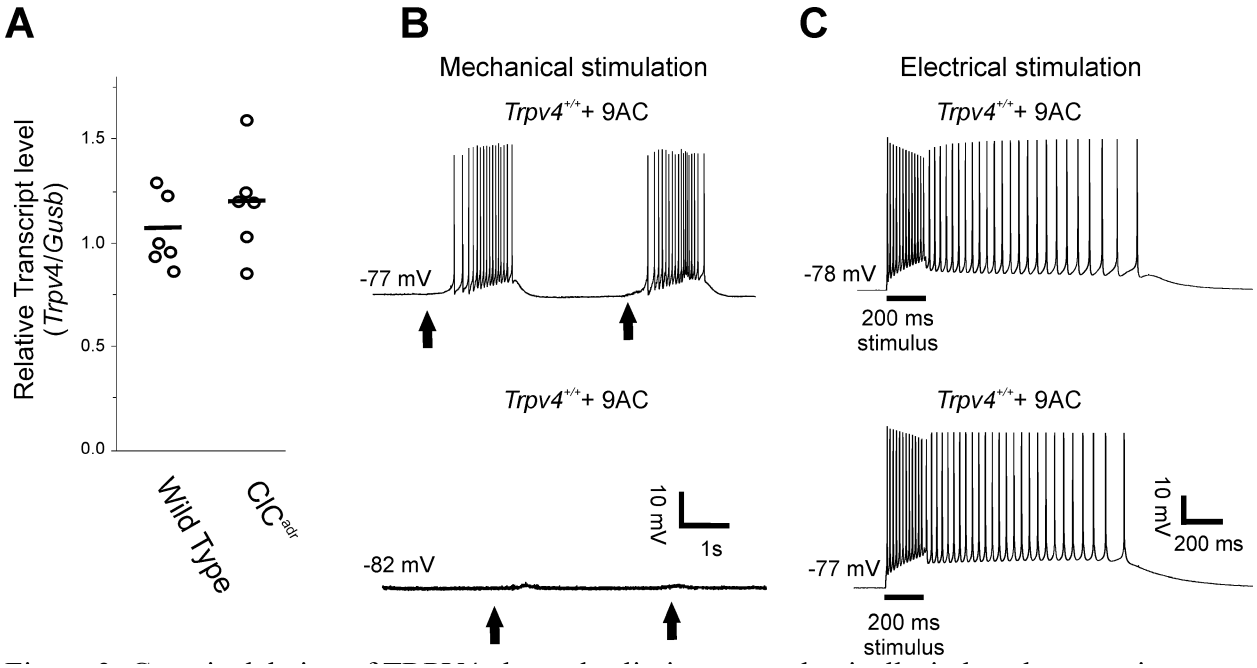

Figure 3:

Genetic deletion of TRPV4 channels eliminates mechanically-induced myotonia without affecting electrically-induced myotonia. A) Scatter plot of the relative *Trpv4* transcript levels normalized to the reference gene *Gusb* for 6 wild type and 6 ClC^{adr} extensor digitorum longus muscles. The horizontal bars represent the mean of each group. B) In 9AC-treated muscle from $Trpv4^{+/+}$ mice, mechanically-induced myotonia was present in 21/28 fibers from 4 mice whereas in 9AC-treated muscle fibers from $Trpv4^{-/-}$ mice, mechanically-induced myotonia was present in 0/28 fibers from 4 mice. At the times indicated by vertical arrows, a blunt electrode was manually advanced to mechanically stimulate the fiber being studied. C) Electrically-induced myotonia was present in 35/35 9AC-treated $Trpv4^{+/+}$ fibers from 4 mice and 22/22 $Trpv4^{-/-}$ fibers from 3 mice.

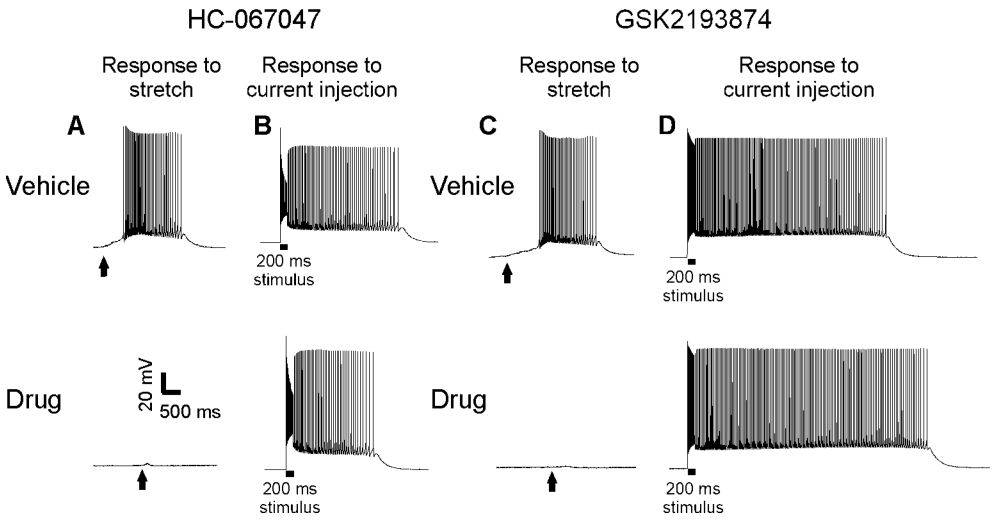

Figure 4:

Treatment with small molecule inhibitors of TRPV4 greatly reduces mechanically-induced myotonia in ClC^{adr} muscle. A) Mechanically-induced myotonia was present in 28/28 vehicle-treated fibers from 4 mice, and 0/28 fibers treated with 1 μM HC-067047 from 4 mice. At the times indicated by vertical arrows, a blunt electrode was manually advanced to mechanically stimulate the fiber being studied. B) Electrically-induced myotonia was present in 33/33 vehicle-treated fibers from 5 mice and 48/48 HC-067047-treated fibers from 5 mice. C) Following application of vehicle 19/20 fibers from 3 mice had mechanically-induced myotonia. After application of GSK2193874, 3/20 fibers from 3 mice had mechanically-induced myotonia. D) Electrically-induced myotonia was present in 21/21 vehicle-treated fibers from 3 mice and 19/19 fibers treated with 1 μM GSK2193874 from 3 mice.

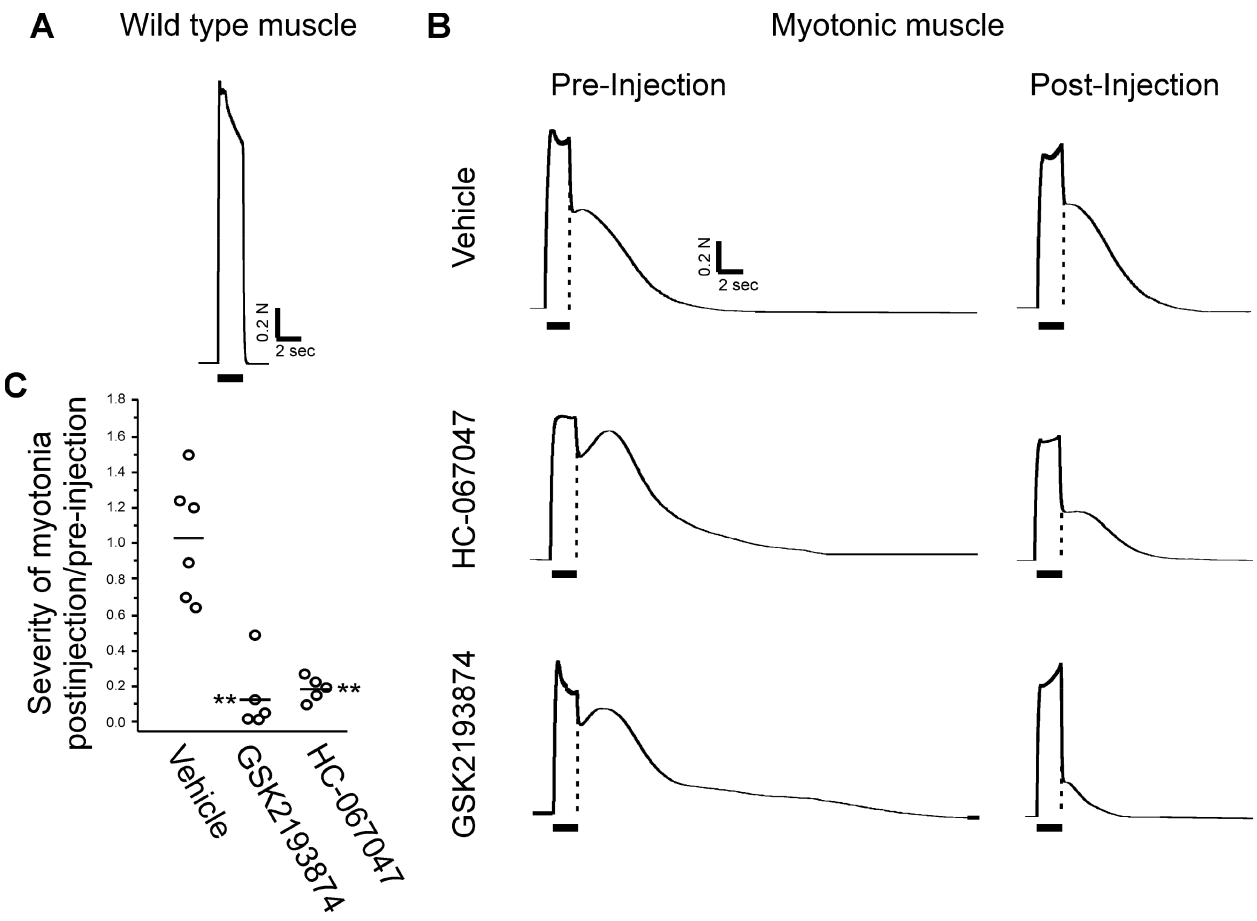

Figure 5:

Block of TRPV4 is effective in treating myotonia *in vivo*. A) The response of wild type muscle to 2 s of 60 Hz stimulation of the sciatic nerve. The horizontal bar under the trace indicates the period of 60 Hz stimulation of the sciatic nerve. Force is fully fused and there is immediate, complete relaxation following termination of stimulation. B) The response of muscle from ClC^{adr} mice to 60 Hz stimulation. The vertical dotted line in each trace represents the rapid relaxation of force that occurs in wild type muscle following termination of nerve stimulation. Pre-injection, force generation during 60 Hz stimulation is fused normally, but relaxation following termination of stimulation is only partial and there is continued contraction for many seconds secondary to myotonia. At 45 minutes post-injection of vehicle, myotonia is minimally changed, but is greatly decreased in mice that received either HC-067047 or GSK2193874. C) Scatter plot of the ratio of severity of myotonia post-injection to severity of myotonia pre-injection in each mouse. The horizontal bars represent the mean of each group. Following injection of vehicle there was no change in severity of myotonia. Following injection of either drug there was a marked reduction. ** indicates $p < 0.01$ versus vehicle.

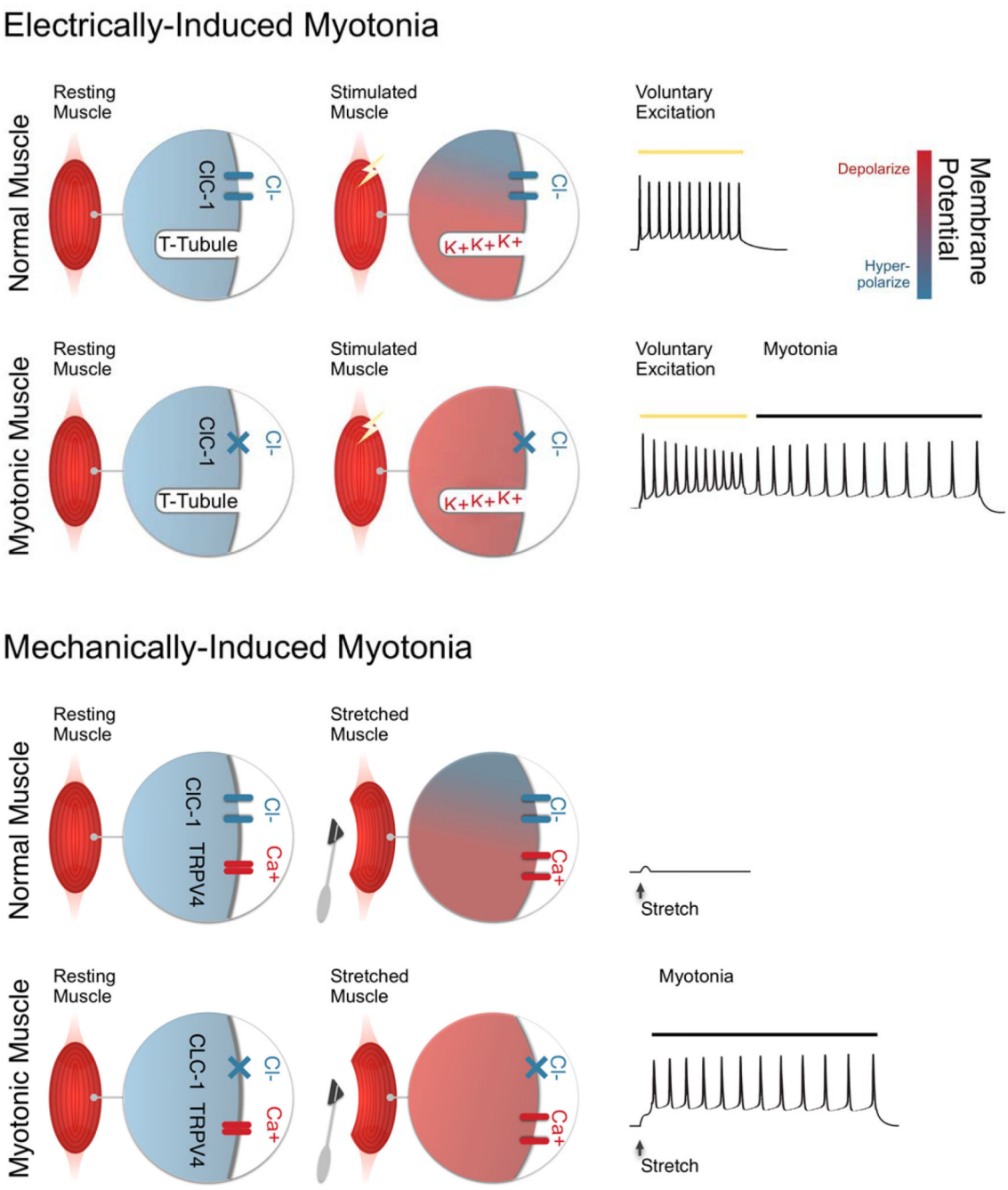

Figure 6:

Distinct mechanisms underlying electrically- and mechanically-induced myotonia. Electrically-induced myotonia (top two rows): in wild type muscle Cl^- current plays a central role in controlling resting potential both at rest and during firing of action potentials during voluntary movement. Because of the stabilizing influence of Cl^- current, K^+ build-up in t-tubules does not cause sufficient depolarization to trigger myotonia following voluntary activation of muscle. In myotonia congenita, muscle Cl^- current is reduced such that K^+ build-up causes depolarization large enough to trigger myotonia. Mechanically-induced myotonia (bottom two rows): mechanical stimulation of wild type muscle activates TRPV4

channels, which depolarizes muscle. This depolarization is opposed by Cl^- current such that myotonia is not triggered. Activation of TRPV4 channels during mechanical stimulation of muscle with reduced Cl^- current causes depolarization sufficient to trigger myotonia.

Table 1

Genetic deletion and pharmacologic inhibition of TRPV4 have no effect on skeletal muscle passive properties and single action potentials

	Resting potential (mV)	Input resistance (MΩ)	Action potential		
			Threshold (mV)	Maximum rate of rise (mV/ms)	Peak (mV)
$TRPV4^{+/+}$ + 9AC	−83.9 ± 0.4	1.17 ± 0.09	−62.7 ± 0.2	239.4 ± 7.6	32.5 ± 0.9
$TRPV4^{-/-}$ + 9AC	−83.6 ± 0.8	1.20 ± 0.07	−62.9 ± 0.7	231.9 ± 2.0	29.6 ± 0.6
ClC^{adr} + vehicle	−80.9 ± 0.5	0.85 ± 0.03	−56.7 ± 0.5	234.1 ± 30.4	30.1 ± 4.7
ClC^{adr} + HC-067047	−80.9 ± 0.5	0.79 ± 0.05	−56.9 ± 0.6	236.6 ± 30.6	29.9 ± 3.9
ClC^{adr} + vehicle	−84.5 ± 0.3	1.06 ± 0.04	−60.4 ± 0.4	244.9 ± 15.0	35.7 ± 1.0
ClC^{adr} + GSK2193874	−82.8 ± 1.4	1.15 ± 0.07	−59.8 ± 1.0	242.8 ± 2.7	36.6 ± 0.7

For studies of HC-067047, n = 28 fibers from 4 muscles for both groups. For studies of GSK2193874, n = 25 fibers from 3 muscles for vehicle and 19 fibers from 3 muscles for drug. For studies of $TRPV4^{-/-}$ mice, n = 39 fibers from 3 $TRPV4^{+/+}$ mice, and 22 fibers from 3 $TRPV4^{-/-}$ mice. Data shown are mean ± SEM. None of the differences between vehicle and drug treatment groups and between TRPV4 genotypes were statistically significant.