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Sodium channel slow inactivation as a therapeutic target for myotonia congenita

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Abstract

Objective—Patients with myotonia congenita have muscle hyperexcitability due to loss-of-function mutations in the chloride channel in skeletal muscle, which causes spontaneous firing of muscle action potentials (myotonia), producing muscle stiffness. In patients, muscle stiffness lessens with exercise, a change known as the warm-up phenomenon. Our goal was to identify the mechanism underlying warm up and to use this information to guide development of novel therapy.

Methods—To determine the mechanism underlying warm-up, we used a recently discovered drug to eliminate muscle contraction, thus allowing prolonged intracellular recording from individual muscle fibers during induction of warm-up in a mouse model of myotonia congenita.

Results—Changes in action potentials suggested slow inactivation of sodium channels as an important contributor to warm-up. These data suggested enhancing slow inactivation of sodium channels might offer effective therapy for myotonia. Lacosamide and ranolazine enhance slow inactivation of sodium channels and are FDA-approved for other uses in patients. We compared the efficacy of both drugs to mexiletine, a sodium channel blocker currently used to treat myotonia. *In vitro* studies suggested both lacosamide and ranolazine were superior to mexiletine. However, *in vivo* studies in a mouse model of myotonia congenita suggested side effects could limit the efficacy of lacosamide. Ranolazine produced fewer side effects and was as effective as mexiletine at a dose that produced none of mexiletine's hypoexcitability side effects.

Interpretation—We conclude ranolazine has excellent therapeutic potential for treatment of patients with myotonia congenita.

Introduction

Myotonia is characterized by slowed muscle relaxation following voluntary contraction and is a common symptom in a family of skeletal muscle channelopathies known as the non-

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dystrophic myotonias. Myotonia congenita is one of the non-dystrophic myotonias and is caused by reduction in muscle chloride conductance stemming from loss-of-function mutations of ClC-1 chloride channels.^{1, 2} Chloride conductance normally accounts for 70%–80% of resting muscle membrane conductance and functions to stabilize the membrane voltage near the chloride equilibrium potential.^{3, 4} In the absence of chloride conductance, muscle becomes hyperexcitable and action potentials occur spontaneously following cessation of voluntary contraction.

A major unsolved question is why repetitive activity in patients with myotonia congenita triggers a reduction in muscle stiffness that is known as the warm-up phenomenon.^{5, 6} Changes in membrane potential, membrane conductance, and slow inactivation of sodium channels have all been proposed to underlie warm-up.^{7, 8} Unfortunately, it has been impossible to directly study the mechanism underlying warm-up because muscle contraction makes it impossible to perform intracellular recording from individual muscle fibers during the stimulation necessary to induce warm-up. Because of this technical challenge, the mechanism underlying warm-up has remained unknown since its original description almost 40 years ago.⁵ A better understanding of the mechanism underlying warm-up could help in development of more effective therapy.

The recent discovery of N-benzyl-p-toluenesulfonamide (BTS), which eliminates muscle contraction with minimal effect on excitability,^{9, 10} has made it possible to perform intracellular recording in muscle fibers during thousands of action potentials.^{11, 12} BTS blocks muscle contraction by inhibiting interaction between myosin and actin, while causing minimal alteration in Ca metabolism or excitability,⁹ such that Ca-dependent processes are unperturbed. Using BTS in a mouse model of myotonia congenita, we were able to observe the electrophysiological correlate of the severe stiffness of myotonia at baseline, perform intracellular recordings, and induce warm-up in isolated muscle fibers in the absence of muscle contraction. This allowed us to test data from studies of mechanisms underlying warm-up and identify a new avenue for therapy. We then tested two potential novel therapies for myotonia congenita both *in vitro* and *in vivo* in a mouse model of the disease.

Methods

Mice

All experiments were performed using a colony of ClCn1^{adr-mto2J} (ClC^{adr}) mice, which have a null mutation in the ClC-1 gene. The mice were obtained from Jackson labs (Bar Harbor, ME) and a breeding colony established. Myotonia was identified clinically in ClC^{adr} mice via myotonic appearance of the animals as previously described.¹³ Asymptomatic littermates were used as controls: two thirds of asymptomatic mice were likely heterozygous for the ClC-1-null mutation. As unaffected littermates have previously been shown not to have myotonia or alteration in macroscopic chloride current, we did not make an effort to distinguish them from wild type mice.^{14, 15} Mice were used starting at 6 weeks and going to 3 months of age.

Intracellular recording and stimulation protocol

Mice were sacrificed using CO₂ inhalation followed by cervical dislocation, and both extensor digitorum longus muscles were dissected out tendon-to-tendon. Experiments were done on the first muscle while the second muscle was maintained in oxygenated solution. Experiments on the second muscle began within 3 hours of killing the mouse. All muscles were maintained and recorded from at 22°C. We have found that muscle electrical properties are stable for up to 6 hours if the muscle is maintained in adequately oxygenated solution. Data from both muscles were pooled and considered one sample for statistical purposes. The recording chamber was continuously perfused with 30 mls of recirculating Ringer solution containing (in mM) NaCl, 118; KCl, 3.5; CaCl₂, 2; MgSO₄, 0.7; NaHCO₃, 26.2; NaH₂PO₄, 1.7; glucose, 5.5 (pH 7.3–7.4, 20–22°C), and equilibrated with 95% O₂ and 5% CO₂.

To prevent contraction of muscle fibers, muscles were loaded with 50μM N-benzyl-p-toluenesulfonamide (BTS, Tokyo Chemical Industry)¹⁰ for at least 30 minutes prior to recording. BTS was dissolved in DMSO and added to perfusate; maximal concentration of DMSO was 0.1%, less than the concentration of 0.15%, which has been found to not affect resting membrane properties of skeletal muscle.¹² Muscles were stained for 3 minutes with 10μM 4-(4-diethylaminostyryl)-N-methylpyridinium iodide (4-Di-2ASP, Molecular Probes) and imaged with an upright epifluorescence microscope (Leica DMR, Bannockburn, IL) as previously described.¹⁶ For *in vitro* drug dosing experiments in CIC^{adr} muscles, muscles were prepared as described above and baseline recordings taken. Then treatment drug was added to recirculating perfusate, as described in the *in vitro* drug study below, and experimental recordings performed.

For all intracellular recordings, muscle fibers were impaled with 2 sharp micro-electrodes filled with 3M KCl solution containing 1 mM sulforhodamine to visualize the electrodes with epifluorescence. Electrode resistances were between 15 and 30 MΩ, and capacitance compensation was optimized prior to recording. To induce the warm-up phenomenon, stimulation at 20Hz was applied for differing lengths of time to trigger 1,000, 2,500, or 5,000 action potentials.

Fiber excitability was classified based on excitability of normal muscle. Normal muscle is able to generate at least 3 action potentials during a 60ms pulse, but does not fire action potentials following cessation of current injection. If a fiber generated 3 action potentials and fired action potentials after cessation of 60 ms current injection in 6/10 trials, it was classified as hyperexcitable. If a fiber was unable to generate 3 action potentials during a 60ms current injection, it was classified as hypoexcitable. If a fiber generated 3 action potentials and fired action potentials after cessation of 60ms current injection in 5/10 trials, it was classified as having normal excitability. In almost all cases fibers either had myotonia on 10/10 trials or 0/10 trials.

Input resistance was measured using a 60ms, 10nA hyperpolarizing current pulse. Action potential data were analyzed using OriginPro 8 (OriginLab Corp). Fibers with resting potentials more depolarized than –75mV were excluded from analysis. Action potential rate of rise (dV/dt) was determined by taking the derivative of voltage with respect to time using

Origin software. Action potential threshold was defined as the voltage at which dV/dt was > 10 mV/ms.

Modeling of Action Potentials

Muscle fiber action potentials were simulated using a cable model consisting of 21 compartments which incorporated passive and active membrane properties as previously described.¹⁷ Briefly, active properties were modeled using Hodgkin and Huxley equations for describing voltage dependent behavior of model membranes,¹⁸ which have been used previously to model muscle fiber action potential.¹⁹ Differential equations were integrated numerically using the Runge-Kutta method to provide starting values for the Adams-Moulton predictor-corrector method,²⁰ with adjustable step sizes of 1 to 4 μ s. Leak conductance was reduced to 1/3 of normal to model the effect of reduced chloride conductance on the membrane time constant as previously reported in CIC^{adr} muscle fibers.¹⁴ Other parameters were adjusted as outlined in Table 1 to obtain a close fit to action potentials recorded from CIC^{adr} fibers.

Treatment of myotonia

Drugs used—Ranolazine and mexiletine were purchased from Sigma-Alrich (St. Louis MO) and were directly dissolved in Ringer solution for *in vitro* experiments or phosphate buffered saline for injection into mice. Premixed lacosamide (10mg/ml) was purchased from UCB (Smyrna GA) and diluted in Ringer for *in vitro* experiments or phosphate buffered saline for intraperitoneal injections.

***In vitro* drug study**—We compared the efficacy of lacosamide and ranolazine in treating myotonia to that of mexiletine, a sodium channel blocker which is the current standard of care.²¹ CIC^{adr} muscle was prepared as described in the intracellular recording protocol, above. Baseline recordings were made in a few fibers before drugs were added to the recirculating perfusate. Drug doses were titrated from a low dosage with modest effect on excitability, to a high dosage which caused hypoexcitability (mexiletine and ranolazine) or which greatly exceeded concentrations used clinically (lacosamide). The following doses were tested (in μ M): Mexiletine 10, 25, 50, and 100; Lacosamide 50, 100, 300, and 600; Ranolazine 5, 25, 50, and 100. Each drug dose was tested in 3 different animals and the data pooled. For each dose, we first qualitatively assessed the presence of spontaneous firing of action potentials at the time of impalement. Next, we quantitatively measured excitability by inducing myotonia via current injection. Fibers were classified as hyperexcitable, normally excitable, or hypoexcitable as described in the intracellular recording protocol, and the number of each class for each dose recorded.

***In vivo* dose-finding study**—Mexiletine has been found to be effective in treating myotonia congenita *in vivo* in CIC^{adr} mice at 5mg/kg and 10mg/kg, via subcutaneous injection.²² Neither ranolazine nor lacosamide have been studied as treatment for myotonia congenita. However, ranolazine has been given daily to mice at a dose of 50mg/kg via intraperitoneal injection,²³ and lacosamide has been given to mice at doses up to 100mg/kg via intraperitoneal injection.²⁴ Based on these studies and our *in vitro* data, we chose the following initial drug doses for our dose-finding study: Mexiletine at 5mg/kg, ranolazine at

12.5mg/kg, and lacosamide at 12.5mg/kg. Drugs were delivered via intraperitoneal injection to 3 treatment groups of 4 mice each, with a fourth control group injected with vehicle. Dosages of each drug were doubled for each successive step in this study. 3 days were given before the next doubled dose was administered, to allow time for drug elimination. All 3 drugs have half-lives significantly less than 1 day in humans;^{25–27} thus, 3 days allowed for more than 5 half-lives of drug decay. Gross motor performance (walking and interacting with the environment) was monitored at each dosage, until dosage resulted in apparent toxicity. For lacosamide, clear sedation and ataxia were apparent at a dose of 50 mg/kg. For ranolazine, sedation and ataxia were apparent at 100 mg/kg. As toxicity was obvious, higher doses of lacosamide and ranolazine were not tested. Mexiletine showed no beneficial effects at 5mg/kg, while possible mild ataxia was apparent at 40 mg/kg. While monitoring motor performance during these trials, we also determined the time-course of each drug's maximal impact. All 3 drugs had positive effects on motor function within 5 minutes of injection and continued to have positive effects for at least 30 minutes.

***In vivo* study of therapeutic drug effects**—Based on data from the dose-finding study, we decided to test 3 dosages of mexiletine (10, 20 and 40 mg/kg), 2 dosages of lacosamide (12.5 and 25 mg/kg), and 3 dosages of ranolazine (12.5, 25 and 50 mg/kg). We treated 16 mice and measured motor performance at both 5min and 15min after injection, with the examiner blind to treatment status. All mice were tested for baseline motor function before each treatment. Motor function was analyzed by timing the righting reflex 3 times^{22, 28} and by scoring 2 performance trials on a modified Rotarod test. In a traditional Rotarod test, mice are placed on a stationary rod and the speed of the rod is gradually increased until the mice can no longer stay on the rod. We found this method gave mice time to warm up and was therefore insensitive to motor dysfunction. To better measure treatment effect on muscle stiffness prior to warm-up, we altered the Rotarod test as follows: Mice were placed on a stationary rod; then the rod was turned on to a single, intermediate speed. Test length was 5 seconds and motor performance was scored on a 0 to 3 scale as follows: 0 for falling off a stationary rod, 1 for falling off as soon as the rod was turned on, 2 for holding on and being rotated for > 5s without walking, and 3 for walking to stay upright for > 5s. After baseline was established, all 16 mice were given the same intraperitoneal treatment and dosage. Motor function was again analyzed at 5min and 15min after injection, which allowed mice sufficient time to recover from any warm-up induced by the baseline trials.

The averaged post-treatment righting times and the averaged post-treatment Rotarod scores (with the 5min and 15min time-points averaged together) were compared to the averaged baseline performance for each mouse. As the same 16 mice were used for all treatment and control groups, at least 3 days were given between studies to allow time for drug elimination, as described in the dose-finding study. As no mouse demonstrated significant variation in baseline motor performance from one pre-treatment trial to another, there was no evidence that previous drug treatment had any lingering effect.

***In vivo* measure of drug impact on myotonia**—The dose of each drug that gave the best improvement in motor performance in the previous trial was selected for this study:

Mexiletine 20 mg/kg, ranolazine 50 mg/kg, and lacosamide 25 mg/kg; plus a saline control - thus 12 total mice were used, 3 for each treatment group. Mice were anesthetized with inhaled isoflurane. Isoflurane appeared to have little effect on myotonia as prolonged and frequent myotonia on EMG was present in all CIC^{adr} mice at baseline. Muscles impaired *in vivo* included the left paraspinal and gastrocnemius muscles prior to drug injection, and the right paraspinal and gastrocnemius muscles after drug injection. Prior to treatment, the baseline degree of myotonia was assigned a subjective severity by 2 blinded EMG examiners using standard EMG technique: 0 = no action potentials firing from needle insertion, 1 = firing upon insertion and subsiding within 2 seconds of needle movement, 2 = firing upon insertion and lasting more than 2 seconds, but subsiding within 10 seconds, 3 = continued spontaneous firing in the absence of needle movement. The severity of myotonia was reassessed 10 minutes after drug injection. Myotonia levels from both paraspinal and gastrocnemius were averaged together. The pre-treatment and post-treatment myotonia averages were compared in each mouse to determine the degree of drug impact on myotonia. After recordings, mice were euthanized by carbon dioxide inhalation, followed by cervical dislocation.

Statistics

For the *in vitro* study of the warm-up phenomenon, each muscle fiber was compared to itself before and after warm-up, and the paired Student's t-test was used, with n as the number of muscle fibers. Data were tested for normal distribution prior to use of the Student's t-test. Bonferroni correction was used for multiple comparisons. For the *in vitro* study of efficacy of drugs, the results of each muscle were averaged together. The mean values for muscles were compared using the student's t-test with n as the number of muscles studied. For the *in vivo* study of drug impact on motor performance, each mouse was compared to itself before and after administration of drug, and the paired Student's t-test was used, with n as the number of mice studied. For the *in vivo* study of drug injection effects on muscle excitability, data from multiple muscles were averaged to give both a pre-treatment and a post-treatment mean value for each mouse, and these means were used for comparison with n as the number of mice studied.

Ethical Approval

All procedures involving mice were approved by the Wright State IACUC committee.

Results

Mice lacking functional CIC-1 chloride channels exhibit warm-up

CICn1^{adr-mto2J} mice (CIC^{adr} mice) that are homozygous for the null mutation in the CIC-1 gene have severe muscle stiffness that manifests as impaired ability to run²⁹, a stiff gait, and an inability to rapidly right after being placed in a supine position. When CIC^{adr} mice were placed in a supine position they initially took 3.5 ± 1.0 seconds (n = 10 mice) to right themselves. With repeated testing over 30s, the mice were able to right themselves within an average of 1.7 ± 0.3 seconds (p < .01). The motor improvement appears similar to the warm-up phenomenon experienced by patients with myotonia congenita. We used CIC^{adr} mice to study the mechanism underlying the warm-up phenomenon.

Warm-up can be induced *in vitro* in CIC^{adr} muscle in the absence of muscle contraction

When extensor digitorum longus muscle fibers from 2–3 month old mice were impaled *in vitro* with sharp electrodes, the electrophysiological correlate of the severe stiffness of CIC^{adr} mice at baseline was easily observed. Impalement triggered spontaneous runs of action potentials, something never seen in normal muscle. After allowing the membrane potential to stabilize, CIC^{adr} muscle fibers stopped firing spontaneously such that a 5 ms current injection of more than 10 nA was necessary to trigger a single action potential. When a 60 ms pulse of 20 nA or more was injected, action potentials continued to fire following termination of current injection (Figure 1). In phenotypically normal, age-matched littermates, action potentials were never observed once stimulation was terminated (n = 6 mice, 30 fibers). The increased excitability in CIC^{adr} mice was accompanied by an increase in input resistance, but characteristics of action potentials were otherwise similar between CIC^{adr} mice and unaffected littermates (Table 2). These findings are similar to those reported previously for CIC^{adr} muscle fibers.¹⁴

Slow inactivation of sodium channels contributes to warm-up of CIC^{adr} muscle *in vitro*

To study the electrophysiological correlate of the reduction in excitability that underlies the warm-up phenomenon, it is necessary to be able to study warm-up in an isolated muscle *in vitro*. Warm-up has been shown to occur *ex vivo* in wild type muscle made acutely myotonic by block of muscle chloride channels.²⁹ To study action potential and passive membrane properties associated with warm-up it is necessary to record from individual muscle fibers before and after repeated activation of the fiber. The recent discovery of N-benzyl-p-toluenesulfonamide (BTS) has made this possible. BTS blocks muscle contraction by inhibiting interaction between myosin and actin, with minimal alteration in Ca metabolism or excitability.^{9, 10} After blocking muscle contraction with BTS, 5000 action potentials were delivered at 20 Hz in skeletal muscle from CIC^{adr} mice using a protocol similar to one that has been used on wild type skeletal muscle fibers.^{11, 12} We examined whether prolonged firing of action potentials led to resolution of myotonia. Myotonia was classified as the continued firing of action potentials following the end of a 60 ms depolarizing pulse. In normal muscle *in vitro*, no action potentials are fired after the depolarizing current is terminated.³ In all muscle fibers from CIC^{adr} mice, myotonia was present at baseline. Following a train of 5000 action potentials, myotonia was eliminated in all 18 fibers studied from CIC^{adr} mice (Figure 1). In 5 fibers, impalement was stable enough to allow for additional recording for 5 minutes following stimulation, and in all these 5 fibers myotonia returned following inactivity (Figure 1). Elimination of myotonia following prolonged firing and the return of myotonia following rest indicates the warm-up phenomenon can be triggered *in vitro* in the absence of muscle contraction. We conclude that warm-up is triggered by action potentials rather than muscle contraction.

The ability to trigger warm-up while recording from an individual muscle fiber allowed for comparison of biophysical properties of individual fibers before and after warm-up, to determine potential mechanisms underlying warm-up. Action potential threshold was elevated in parallel with resolution of myotonia (Table 3). This demonstrated that resolution of myotonia with warm-up is paralleled by reduced excitability of fibers. A number of mechanisms might contribute to reduced excitability following warm-up. One proposed

mechanism for warm-up is accumulation of K^+ in the t-tubules, which depolarizes the muscle and increases Na^+ channel inactivation.⁷ Alternatively, K^+ accumulation could stimulate the Na^+-K^+ pump which, because of its electrogenicity, might hyperpolarize the membrane to eliminate myotonia.³⁰ It is known that a brief period of muscle fiber depolarization occurs following 5–10 action potentials in myotonic muscle.³ We examined whether prolonged depolarization or hyperpolarization occurred after 5000 action potentials. No change in resting potential occurred in parallel with warm-up (Table 3). These data suggest the mechanism underlying reduced excitability following warm-up does not involve a change in resting potential.

Another potential mechanism underlying warm-up is an increase in resting membrane conductance.³¹ In wild type rat muscle, it was found that prolonged firing of action potentials triggered a marked increase in membrane conductance.^{11, 12} While it appeared that most of the increase in conductance was mediated by ClC-1 chloride channels, there was also increased potassium conductance.^{11, 12} To measure changes in resting membrane conductance, a 60ms hyperpolarizing pulse was given to measure input resistance. While there was a statistically significant decrease in input resistance following 5000 action potentials (Table 3), the change was relatively small and, as described in the next section, seems unlikely to be a major contributor to the reduction of excitability that underlies warm-up.

Another mechanism that might underlie warm-up is slow inactivation of sodium channels.⁸ The relative density of sodium channels opening during the upstroke of the action potential can be estimated by measuring the maximal rate of action potential rise and action potential peak.³² Thus, if slow inactivation of sodium channels contributes to warm-up, both action potential rate of rise and action potential peak will be reduced. When the action potential rate of rise and action potential peak were measured following warm-up, both were reduced (Figure 2, Table 3). These data are consistent with slow inactivation of sodium channels following warm-up.

Modeling the change in excitability following warm-up

To determine the relative importance of increased leak conductance versus slow inactivation of sodium channels in reducing excitability following warm-up, action potentials were simulated using our previously published model.¹⁷ Before attempting to model changes induced by warm-up we adjusted parameters to optimize the model's match to action potentials measured from ClC^{adr} muscle fibers at baseline. Achieving a good fit at baseline required increases in the forward rate of activation of both sodium (α_m) and potassium (α_n) channels (Table 1). Without the increases in rates of activation, action potentials were too broad. Accurate modelling of the relatively low action potentials peaks in ClC^{adr} muscle (+10 to +15 mV, Table 2 and ¹⁴) required reduction of the sodium equilibrium potential from +40 mV to +30 mV. Using the modified values, we were able to model an action potential waveform that closely resembled the measured ClC^{adr} waveform (Fig 3A).

Measured changes following warm-up included reduction in the rate of rise, reduction in the peak potential reached, reduction in the rate of repolarization, as well as a small, sustained depolarization lasting 5 to 10ms after the peak (Fig 2, Fig 3B). Through simulation, we

explored the potential contribution of increased leak conductance and found a 15% increase in leak (to mimic the reduction in input resistance, Table 3) had minimal effect on action potential morphology (Fig 3C). The easiest way to simulate reduction in rate of action potential rise and peak potential was reducing available sodium conductance (G_{Na}) by 40% (Fig 3D). However, reduction of G_{Na} alone did not recreate the reduction in rate of repolarization of the action potential. To simulate the slowed repolarization, it was necessary to reduce the maximal conductance of voltage activated K channels (G_K , Fig 3E). While the reduction of G_K alone allowed for accurate simulation of the early part of repolarization, it did not allow accurate simulation of the small, sustained depolarization that remained 5 to 10ms after the action potential peak. To simulate this depolarization, it was necessary to include a 5mV depolarized shift in the K equilibrium potential (E_K , Fig 3F). Our findings suggest warm-up may be accompanied by reduction in both voltage-activated G_{Na} and G_K as well as a depolarized shift in E_K .

The time course of warm-up parallels the time course of sodium channel slow inactivation

In skeletal muscle there is a very slow form of sodium channel slow inactivation that has a time constant on the order of minutes.^{33–35} If slow inactivation of sodium channels contributes to warm-up, the duration of warm-up should parallel the degree of sodium channel slow inactivation. We measured the reduction in rate of action potential rise to estimate the relative reduction in density of functional sodium channels to determine whether it correlated with duration of warm-up. Different durations of stimulation at 20 Hz were used to induce warm-up. After 1,000 action potentials, rate of action potential rise was reduced by $15.1 \pm 3.3\%$ relative to baseline ($n = 10$ fibers); after 2,500 action potentials it was reduced by $18.2 \pm 2.6\%$ ($n = 11$ fibers), and after 5,000 action potentials it was reduced by $40.2 \pm 3.7\%$ ($n = 16$ fibers). All three durations of stimulation eliminated myotonia in 100% of fibers, but the duration of warm-up varied in parallel with the reduction in rate of action potential rise (Figure 4). Following 1,000 action potentials, myotonia had returned in 100% of fibers 1 minute following termination of stimulation. After 2,500 action potentials, it took 5 minutes; and after 5,000 action potentials, it took 8 minutes. The parallel between the degree of reduction in rate of action potential rise and the duration of warm-up is consistent with possibility that slow inactivation of sodium channels is an important contributor to warm-up.

Increasing slow inactivation of sodium channels to treat myotonia of CIC^{adr} muscle in vitro

If slow inactivation of sodium channels underlies warm-up, it might be possible to mimic warm-up with drugs that increase sodium channel slow inactivation. There are two drugs that are FDA approved, which increase slow inactivation of sodium channels. One of the drugs (lacosamide) is used to treat epilepsy,³⁶ while the other drug (ranolazine) is used to treat myocardial ischemia.³⁷ Both lacosamide and ranolazine increase slow inactivation by inducing a hyperpolarized shift in the voltage dependence of slow inactivation.^{38–42} We compared the *in vitro* efficacy of lacosamide and ranolazine in treating myotonia to mexiletine, a sodium channel blocker that is the current standard of care.²¹ Drug doses were titrated from low dosage with modest effect on excitability to high dosage which caused hypoexcitability (mexiletine and ranolazine) or which greatly exceeded concentrations used clinically (lacosamide).

We measured the effect of drugs on hyperexcitability of CIC^{adr} muscle fibers in two ways. The first was a qualitative evaluation of the presence of spontaneous firing of action potentials at the time of impalement (impalement myotonia). Normal muscle does not fire action potentials when it is impaled, but all CIC^{adr} fibers have impalement myotonia. None of the three drugs eliminated impalement myotonia, except at high doses when muscle became hypoexcitable (see below). The second measure of excitability was a quantitative evaluation of myotonia induced by current injection. Normal muscle fires repetitively during current injection, but firing stops as soon as current injection is terminated (Figure 1). In CIC^{adr} muscle, 100% of fibers continue to fire action potentials after current injection is terminated. Fibers were classified as hyperexcitable, normally excitable, or hypoexcitable as described in Methods and shown in Figure 5A. There was no dose of mexiletine that normalized excitability in a majority of fibers (Figure 5). At 10 and 25 μ M of mexiletine, most fibers remained hyperexcitable; at 50 μ M there was a wide range in excitability; and at 100 μ M almost all fibers were hypoexcitable. Lacosamide was effective in normalizing excitability, but only at a dose of 600 μ M. Ranolazine normalized excitability in 100% of fibers at a dose of 50 μ M and induced hypoexcitability at a dose of 100 μ M.

Increasing slow inactivation of sodium channels improves motor performance of CIC^{adr} mice

We next studied the relative efficacy of mexiletine, lacosamide, and ranolazine in CIC^{adr} mice *in vivo*. We used previous studies^{22–24} as well as our *in vitro* data to guide initial choices of drug doses and performed a dose escalation study as described in Methods. All three drugs improved motor function as measured by the decrease in time of the righting reflex (Figure 6). There was no statistically significant difference in efficacy between any of the highest doses of drugs on this measure of motor function. At their optimal doses, both mexiletine and ranolazine triggered greater improvement on the Rotarod than lacosamide ($p < .05$). At 40 mg/kg of mexiletine, some sedation and ataxia were present and this appeared to account for the decline in Rotarod performance.

We next measured the efficacy of each drug in treating myotonia *in vivo*. Mice were anesthetized with isoflurane and the average duration of myotonia following needle movement in the paraspinal and gastrocnemius muscles was rated on a scale of 0 to 3 by two EMG examiners blinded to the drug given. The drug dose that gave the best improvement in motor performance in the previous study (mexiletine 20 mg/kg, lacosamide 25 mg/kg, and ranolazine 50 mg/kg) was then administered; 10 minutes later, the degree of myotonia was again assessed by the same blinded reviewers. Both lacosamide and ranolazine appeared to be more effective in shortening the duration of myotonia than mexiletine. Lacosamide led to an improvement of 1.2 ± 0.2 on the scale of myotonia duration and ranolazine led to an improvement of 1.0 ± 0.1 , while mexiletine only led to an improvement of 0.4 ± 0.2 and saline led to no improvement ($n = 3$ mice for each drug). Our *in vivo* studies suggest ranolazine is at least as effective in treating motor dysfunction and myotonia in CIC^{adr} mice as either mexiletine or lacosamide.

Discussion

Analysis of the mechanism underlying improved motor performance with exercise (warm-up) in the CIC^{adr} mouse model of myotonia congenita suggested that slow inactivation of sodium channels is an important contributor. The results raised the possibility that enhancing sodium channel slow inactivation might provide effective therapy. Ranolazine and lacosamide are two drugs that increase sodium channel slow inactivation. The efficacy of both drugs in lessening myotonia and improving motor performance was tested both *in vitro* and *in vivo* in CIC^{adr} mice and was compared to the efficacy of the current standard of care: sodium channel blockade with mexiletine. Both ranolazine and lacosamide were more effective than mexiletine in treating myotonia *in vitro*, but sedation and ataxia limited the efficacy of lacosamide *in vivo*. Ranolazine appeared to be as good, or better, than mexiletine on all *in vivo* measures of motor performance and myotonia in CIC^{adr} mice.

Mechanisms contributing to warm-up

Ours is the first study to determine the changes in excitability that occur during warm-up. The first change identified was reduction of input resistance. Reduction in input resistance in wild type muscle occurs following prolonged firing due to activation of both K_{ATP} and CIC-1 chloride channels.^{11, 12} As CIC^{adr} muscle lacks functional CIC-1 channels,^{1, 2} the decrease in input resistance in CIC^{adr} muscle is likely due to activation of K_{ATP} channels. While our simulation of action potentials suggests activation of K_{ATP} channels has little effect on action potential waveform, activation of these channels may contribute to warm-up by increasing the amount of current required to reach threshold for generation of action potentials.

A depolarized shift in K equilibrium potential caused by t-tubular K accumulation following repetitive firing has been suggested as the source of depolarization leading to repetitive firing during myotonia caused by reduction of muscle chloride conductance.³ Although we found that warm-up was not accompanied by a change in resting potential, it was necessary to include a 5mV depolarized shift in the equilibrium potential for K following warm-up to accurately simulate the changes in action potential waveform. The magnitude of this depolarization in K equilibrium potential is similar to that predicted by simulation of K accumulation in the t-tubules during repetitive firing.^{43, 44} As CIC^{adr} muscle has low Cl conductance, what is offsetting the depolarized shift in K equilibrium potential such that membrane potential is not depolarized? In rat soleus muscle, increased $\text{Na}^+\text{-K}^+$ pump activity following stimulation provides enough current to cause a 10 mV hyperpolarization of the resting potential.⁴⁵ We hypothesize that, during prolonged firing of CIC^{adr} muscle, increased activity of the $\text{Na}^+\text{-K}^+$ pump offsets a depolarized shift in the equilibrium potential for K, such that there is little net change in resting potential.

The most dramatic changes in action potential waveform following warm-up were reduction in the rate of action potential rise and decay as well as a nearly 10mV reduction in peak amplitude. Simulation of these changes was best achieved by reduction of voltage-activated Na conductance as well as voltage-activated K conductance. There is a process of slow inactivation of K channels which has a time course of seconds;^{46, 47} and since we measured action potential waveform within seconds of warm-up, it is possible that slow inactivation

underlies the reduction in voltage-activated K conductance suggested by our simulations. However, it seems unlikely that reduction in voltage-activated K conductance plays an important role in warm-up, as its only effect is to cause a slight increase in action potential half-width. As there is a greater than 20ms delay between action potentials during myotonia in CIC^{adr} muscle, a slight widening of action potential half-width seems unlikely to have a significant effect on spontaneous firing.

The only way we found to accurately simulate the reduction in rate of rise and peak of the action potentials following warm-up was to include slow inactivation of sodium channels. Slow inactivation of Na channels in skeletal muscle has a time constant on the order of minutes.^{33–35} This is similar to the time course of induction and recovery from warm-up. By reducing the density of sodium channels available to open, slow inactivation causes the action potential threshold to move towards more depolarized potentials such that excitability is reduced.

Enhancing sodium channel slow inactivation to treat myotonia

There is currently no FDA approved treatment for myotonia congenita.⁴⁸ The most commonly used strategy to treat myotonia is to reduce muscle sodium currents.^{28, 48} Our inference that slow inactivation of sodium channels contributes to elimination of myotonia during warm-up suggested that enhancing slow inactivation of sodium channels might offer a more physiologic approach to reducing muscle sodium current. Ranolazine and lacosamide are FDA approved drugs whose mechanism of action is an increase in sodium channel slow inactivation.^{38–42} We compared the efficacy of ranolazine and lacosamide, both *in vitro* and *in vivo*, to mexiletine, which is the current standard therapy for myotonia congenita.^{21, 48}

In vitro studies of muscle from CIC^{adr} mice suggested ranolazine and lacosamide were more effective than mexiletine in normalizing muscle excitability. In agreement with this finding, ranolazine and lacosamide appeared more effective than mexiletine in reducing the duration of myotonia *in vivo*. Reduction in the duration of myotonia following treatment with lacosamide and ranolazine makes sense as both drugs function to increase slow inactivation, which accumulates during repetitive firing to end the myotonic runs of action potentials. However, there is no obvious reason that mexiletine should be any less effective in shortening the duration of myotonia. Its mechanism of action is a use-dependent reduction in sodium current that is mediated by a hyperpolarized shift in the voltage dependence of fast inactivation.⁴⁹ However, despite mexiletine's inferiority in lessening myotonia *in vitro* and *in vivo*, it was superior to lacosamide in improving motor function *in vivo*. This may be due the propensity of lacosamide to cause ataxia and vertigo.⁵⁰ These findings in mice raise the possibility that side effects of lacosamide may limit its efficacy in treating myotonia in patients.

In CIC^{adr} mice, ranolazine appeared to cause less sedation and ataxia than lacosamide and was as good, or better, than mexiletine in all *in vitro* and *in vivo* measures of improvement in myotonia and motor function. It is not immediately obvious why reduction of sodium current due to a hyperpolarized shift in the voltage dependence of slow inactivation (ranolazine) should be more effective in improving motor function than reduction of sodium current due to a hyperpolarized shift in the voltage dependence of fast inactivation

(mexiletine). We propose that ranolazine may be superior to mexiletine in treating myotonia congenita for three reasons. The first is that since mexiletine modulates an essential function of sodium channels (fast inactivation) it may cause more problems with normal regulation of excitability. In contrast ranolazine slowly modulates the pool of available channels (via slow inactivation) and thus may offer a way of turning the sodium current up or down without affecting channel function. The second advantage of using ranolazine may be that it is less likely to induce loss of muscle fiber excitability at high doses. Mexiletine and other sodium channel blockers can readily reduce sodium current to near zero,^{28, 49, 51} which induces loss of muscle excitability and weakness. Ranolazine reduces sodium current due to a hyperpolarized shift in the voltage dependence of slow inactivation of channels, but the voltage dependence of $Na_v1.4$ slow inactivation is shallow such that slow inactivation is almost never complete (^{33, 38}, see however, ³⁵). Thus ranolazine will not reduce sodium current to near zero unless there is prolonged, severe depolarization of muscle fibers. Because of this difference in mechanism, ranolazine may be less prone to induction of muscle weakness than mexiletine. The third advantage of using ranolazine is that mexiletine is not well tolerated by many patients due to GI side effects and a possible increase in mortality.⁴⁸ We conclude ranolazine has excellent therapeutic potential for treatment of patients with myotonia congenita.

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Figure 1.

Induction of reversible warm-up during intracellular recording: Shown on the left is the response of a normal skeletal muscle fiber to a 60 ms injection of depolarizing current. The fiber is able to repeatedly fire action potentials during the current injection, but firing stops immediately after termination of current injection. The three traces on the right are from an individual CIC^{adr} muscle fiber at baseline, after warm-up, and 5 minutes after warm-up. At baseline the CIC^{adr} fiber is hyperexcitable and continues to fire action potentials after termination of the current injection. After 5000 action potentials have been triggered to induce warm-up, excitability of the fiber has normalized such that no action potentials are fired after termination of current injection. Following 5 minutes of rest, hyperexcitability has returned such that action potentials continue to be fired after termination of current injection. AP = action potential, min = minutes.

Figure 2.

Alteration of the action potential waveform induced by warm-up: Shown are three superimposed action potential traces from an individual muscle fiber before and after 5000 action potentials, and again after 5 minutes of inactivity. Following 5000 action potentials, there is slight hyperpolarization of resting potential, slight elevation of threshold, reduction in both rate of rise and peak of the action potential, as well as slowing of repolarization. Following 5 minutes of inactivity, the action potential has recovered to closely resemble its initial waveform.

Figure 3.

Simulation of action potentials following warm-up is consistent with reduction of voltage gated Na and K conductance as well as a depolarized shift in K equilibrium potential. A) Shown superimposed are an action potential recorded from a ClC^{adr} muscle fiber (black, baseline) and a simulated action potential (red). In the simulated fiber, 15nA of current injection was required to reach threshold. B) Shown are superimposed action potential traces from an individual muscle fiber before (black, same trace as in A) and immediately after 5000 action potentials to induce warm-up (red). C–F: Shown in black in all 4 traces is the same simulated action potential shown in red in A. C) Superimposed on the simulated baseline action potential trace is the simulated action potential when leak conductance is increased by 15%. The traces are so similar that the red trace almost completely obscures the black trace. D) Superimposed (in red) on the baseline simulated trace is the simulated trace when G_{Na} is reduced by 40%. E) Superimposed (in red) on the simulated baseline trace is the trace resulting from a 40% reduction in G_{Na} and a 70% reduction in G_K . F) Superimposed (in red) is the trace resulting from a 40% reduction in G_{Na} , a 70% reduction in G_K and a 5mV depolarization of E_K .

Figure 4.

The duration of warm-up depends on the duration of stimulation. Shown is a plot of the percent of fibers in which myotonia returned at various times following termination of stimulation at 20 Hz. AP = action potential.

Fig 5.

Lacosamide and ranolazine are more effective than mexiletine in normalizing excitability of CIC^{adr} muscle. A) Shown are the responses of 3 different CIC^{adr} muscle fibers to a 60ms injection of depolarizing current following treatment with mexiletine. In the trace on the left, the fiber fired normally during current injection, but was hyperexcitable and fired an additional action potential following termination of current injection. In the trace in the middle, the fiber had normal excitability and was able to fire repetitively during current injection, but immediately stopped firing after termination of current injection. In the trace on the right, the fiber was hypoexcitable and unable to fire repetitively during the 60 ms current injection. B) Plotted for each drug is the percent of hyperexcitable (black), normally excitable (dark grey) and hypoexcitable (light grey) fibers for each dose of drug tested. The bar graph for each drug dose is based on at least 22 fibers from 3 different animals.

Figure 6.

Mexiletine and ranolazine cause greater improvement in motor function than lacosamide. Shown on the left is a plot of the improvement in time of the righting reflex in mice 5 to 15 minutes following intraperitoneal injection of the doses indicated of mexiletine, lacosamide and ranolazine. The highest dose of all three drugs led to statistically significant improvement relative to saline injection (mexiletine $p < .01$, lacosamide $p < .05$, ranolazine $p < .01$). On the right is a plot of the improvement on the Rotarod test of motor performance (see methods for details of scoring). Both mexiletine (20 mg/kg) and ranolazine (50 mg/kg) caused significant improvement in Rotarod function ($p < .01$) while lacosamide did not cause significant improvement relative to saline. $N = 16$ mice for all studies.

Table 1Parameters used to model CIC^{adr} action potentials.

Parameter / units	Previously used values ¹⁷	Values used to model CIC ^{adr} action potentials
$\alpha_m / \text{ms}^{-1}$	1.0	2.0
β_m / ms^{-1}	2.0	2.0
Midpoint of NaCh activation / mV	-37	-39
$\alpha_h / \text{ms}^{-1}$	.0081	.012
β_h / ms^{-1}	8.0	8.0
Midpoint of NaCh inactivation / mV	-73	-77
$\alpha_n / \text{ms}^{-1}$	.020	.080
β_n / ms^{-1}	.067	.067
Midpoint of K activation / mV	-39	-39
Leak conductance / mS/cm ²	.25	.08
Maximum Na conductance / mS/cm ²	500	140
Midpoint of NaCh slow inactivation / mV	-87	-87
Slope of slow inactivation / mV	9.8	9.8
Maximum K conductance / mS/cm ²	30	18
Resting potential (R_m) / mV	-85	-80
$E_{\text{Na}} / \text{mV}$	40	30
E_{K} / mV	-85	-80 to -75

Table 2

	Resting potential (mV)	Threshold (mV)	Action Potential rate of rise (mV/ms)	Action Potential Peak (mV)	Action Potential Half-width (ms)	Input Resistance (MΩ)
Control	-82.2 ± 1.4	-61.6 ± 1.0	299.1 ± 16.0	12.9 ± 1.8	1.39 ± 0.06	0.85 ± 0.05
ClC ^{adr}	-82.1 ± 0.8	-61.7 ± 0.7	235.6 ± 19.1	11.9 ± 1.7	1.25 ± 0.04	1.79 ± 0.11**

All values are shown ± SEM.
** p < .01, n = 6 control mice (30 fibers), 8 ClC^{adr} mice (46 fibers)

Table 3

	Resting potential (mV)	Threshold (mV)	Action Potential rate of rise (mV/ms)	Action Potential Peak (mV)	Action Potential Half-width (ms)	Input Resistance (MΩ)
Baseline	-80.5 ± 0.6	-60.6 ± 0.5	256.4 ± 18.9	14.2 ± 1.3	1.16 ± 0.03	1.69 ± 0.07
5000AP	-81.5 ± 1.0	-56.3 ± 0.7*	149.4 ± 11.0*	4.4 ± 1.8*	1.54 ± 0.05*	1.45 ± 0.06*

The values shown are from 18 C^{adfr} muscle fibers at baseline and after warm-up induced by 5,000 action potentials.

* p < .01 using a paired t-test, n = 18 fibers. All values are shown ± SEM.