CHLORIDE CHANNELS IN MYOTONIA CONGENITA ASSESSED BY VELOCITY RECOVERY CYCLES

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ABSTRACT: *Introduction:* Myotonia congenita (MC) is caused by congenital defects in the muscle chloride channel CLC-1. This study used muscle velocity recovery cycles (MVRCs) to investigate how membrane function is affected. *Methods:* MVRCs and responses to repetitive stimulation were compared between 18 patients with genetically confirmed MC (13 recessive, 7 dominant) and 30 age-matched, normal controls. *Results:* MC patients exhibited increased early supernormality, but this was prevented by treatment with sodium channel blockers. After multiple conditioning stimuli, late supernormality was enhanced in all MC patients, indicating delayed repolarization. These abnormalities were similar between the MC subtypes, but recessive patients showed a greater drop in amplitude during repetitive stimulation. *Conclusions:* MVRCs indicate that chloride conductance only becomes important when muscle fibers are depolarized. The differential responses to repetitive stimulation suggest that, in dominant MC, the affected chloride channels are activated by strong depolarization, consistent with a positive shift of the CLC-1 activation curve.

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Myotonia congenita (MC) is an hereditary disorder characterized by delayed muscle relaxation and stiffness after voluntary activation. It is caused by mutations of the *CLCN1* gene, which encodes the skeletal muscle chloride channel CLC-1. Over 100 different *CLCN1* mutations causing myotonia have been described.[1] Inheritance is autosomal dominant or recessive; in general, autosomal recessive myotonia congenita (ARMC), or Becker myotonia, is more severe clinically than autosomal dominant

myotonia congenita (ADMC), or Thomsen disease. However, mutation-specific variations in phenotype are well recognized, and phenotypic variation even within the same family can be wide,[2] suggesting that epigenetic factors may be involved.

In both ARMC and ADMC, loss of function mutations affecting the CLC-1 channel result in a reduction in chloride conductance.[3] Myotonic discharges are thought to arise because a cumulative afterdepolarization, or depolarizing afterpotential, which occurs with repetitive activity, becomes large enough to trigger self-maintaining activity.[4] This depolarizing afterpotential was shown to arise in the t-tubule system and was attributed to accumulation of potassium ions. In normal muscle fibers it is kept to safe limits by high chloride conductance. The chloride conductance provides an electrical buffering effect, which helps keep the membrane potential close to its resting level, as chloride ions are distributed almost passively and the chloride equilibrium potential is therefore close to the resting potential.[5]

Although studies of nerve membrane excitability are now well established, until recently, *in vivo* studies of muscle excitability have proven more problematic. Previously described single-fiber methods were time-consuming and exhibited considerable variability.[6] In 2009, Bostock and Z'Graggen developed a modified technique, which measures the velocity recovery cycles of a group of adjacent muscle fibers.[7] These multifiber recordings have been applied successfully in several clinical conditions including renal failure,[8] critical illness myopathy,[9] and muscle ischemia secondary to postural hypotension.[10] We have recently performed additional studies to assess the validity of this method for testing muscle membrane properties and found the method to be robust.[11] This technique is now being applied to patients with a variety of channelopathies affecting muscle function. For this purpose, the scope of the tests has

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Pt	Diagnosis	Amino acid change	Gender	Age (y)	Clinical features (Y/N)		
					TW	Falls	Painful stiffness
1	ADMC	Gly230Glu	W	55	N	N	N
2	ADMC	Gly230Glu	M	63	N	N	N
3	ADMC	Gly230Glu	W	46	N	N	N
4	ADMC	Ala313Val	W	38	N	N	N
5	ADMC	Ala313Val	M	72	N	N	N
6	ADMC	Ala313Thr	W	39	N	N	N
7	ADMC	Gly551Asp	M	33	N	N	N
8	ARMC	Val327Ile (+); Arg894X	M	51	Y	Y	N
9	ARMC	c.180+3 A>T (+); Gly190Arg	W	54	Y	N	N
10	ARMC	c.180+3 A>T (+); Gly190Arg	M	50	Y	N	N
11	ARMC	Gly285Glu†	M	23	N	N	N
12	ARMC	Glu624fs†	M	43	N	N	Y
13	ARMC	Arg105Cys; Phe167Leu (+) Glu624fs	M	24	N	N	N
14	ARMC	c.180+3 A>T homozygous	M	49	N	N	Y
15	ARMC	c.1471+1 G>A (+); Arg894X	W	43	N	N	N
16	ARMC	Homozygous intronic mutation	M	67	N	Y	N
17	ARMC	Gly276Ser (+); Pro480fs	M	33	N	Y	Y
18	ARMC	Gly285Glu (+); Met485Val	W	48	N	N	N

Pt, patient; ADMC, autosomal dominant myotonia congenita; ARMC, autosomal recessive myotonia congenita; TW, transient weakness.

†Considered to be a recessive mutation based on family and functional studies. This patient has had the entire coding sequence of the CLCN1 gene (encompassing consensus splice sites) analysed along with rearrangement analysis to detect exonic deletion/duplications, and only this mutation was detected. We cannot exclude mutations in regulatory sequences elsewhere within the gene.

been broadened by adding 2 repetitive stimulation protocols,[12] because muscle activation is known to precipitate muscle dysfunction in some muscle disorders.[13–15] The first of these studies described altered muscle membrane function in patients with Andersen–Tawil syndrome, a rare type of periodic paralysis due to defective inward rectifier channels.[16]

In this study, multifiber muscle velocity recovery cycles (MVRCs) and a repetitive stimulation protocol[12,16] were used to explore the characteristics of the excitability properties of the sarcolemma in patients with MC and to determine whether the excitability measures were helpful in understanding the difference between the ADMC and ARMC subtypes. The early and late phases of supernormality in MVRCs are thought to be related directly to the early and late depolarizing afterpotentials of muscle fibers[7,17]; therefore, it was anticipated that MVRCs would provide a good indication of the exaggerated afterpotentials responsible for myotonic discharges in MC patients.

METHODS
Patients. All 18 patients had chloride channel mutations and were classified as having myotonia congenita (MC); they were aged 46.6 ± 13.8 years (mean $\pm$ SD), range 23 to 72 years (Table 1). Of these 18 patients, 7 were classified as autosomal dominant (ADMC; patients 1–7 in Table 1) and 11 as autosomal recessive (ARMC; patients 8–18), based on a combination of family history and the results of genetic analy-

ses. Several of the patients were being treated with sodium channel modulators (mexiletine, carbamazepine, quinine sulfate) to help control their myotonia (Table 2). To enable testing for possible effects of drug treatment on muscle properties, the patients were divided into those considered on-treatment (designated Rx^+) and those considered off-treatment (Rx^-, i.e., untreated or last dose >5 half-lives of the drug before testing). Six of the ARMC and 5 of the ADMC patients were in the Rx^- category.

Controls. The activity-dependent velocity changes were compared with recordings from 30 normal controls (NC): 19 women and 11 men, aged 41.9 ± 14.7 years (range 21–67 years), including 20 from a previous study.[12]

Consent. Informed written consent was obtained from all patients and controls according to the Declaration of Helsinki. This study was approved by the research ethics committee of St Thomas' Hospital, London, UK, and Kantonal Ethikkommission, Bern, Switzerland.

Study Protocol. All patients had muscle velocity studies; the short exercise test at room temperature; and a blood sample for electrolytes, calcium, and magnesium taken at the end of the session (within 2 h of the studies). These patients had already had routine nerve conduction studies and muscle sampling performed for diagnostic

Pt	Diagnosis	SET pattern*	SET RT abnormal (Y/N)[†]	Maximum amplitude decrement %	Medication[‡]	Rx[‡]
1	ADMC	2	Y	95	(Mexiletine)	−
2	ADMC	3	N	0	Mexiletine	+
3	ADMC	3	N	0	None	−
4	ADMC	2	N	5	None	−
5	ADMC	3	N	0	Mexiletine	+
6	ADMC	2	Y	28	None	−
7	ADMC	3	N	0	None	−
8	ARMC	2	Y	45	(Quinine sulfate)	−
9	ARMC	2	Y	71	Mexiletine	+
10	ARMC	2	Y	69	Mexiletine	+
11	ARMC	3	N	7	Mexiletine	+
12	ARMC	2	Y	12	(Mexiletine)	−
13	ARMC	2	Y	25	(Mexiletine)	−
14	ARMC	2	Y	39	Carbamazepine, quinine sulfate	+
15	ARMC	2	Y	35	None	−
16	ARMC	2	Y	40	Mexiletine	+
17	ARMC	2	Y	25	None	−
18	ARMC	2	Y	31	None	−

Pt, patient; ADMC, autosomal dominant myotonia congenita; ARMC, autosomal recessive myotonia congenita; SET, short exercise test; RT, room temperature; Y, yes; N, no.

*SET pattern as described in Fournier et al.[14] Pattern 1: decrement without recovery during the 60s rest period, typically worsening with consecutive exercise trials; pattern 2: Transient decrement with recovery during each 60s rest period; often becoming less marked with subsequent exercise trials; pattern 3: no decrement.

[†]Upper limit of normal for amplitude-only decrement at room temperature = 11.5% (Tan et al.[15])

[‡]Medication in parentheses and Rx designated (−) if the medication had been omitted for >5 times the half life ($T_{1/2}$) of the drug at the time of the study ($T_{1/2}$ mexilitine 10−12 h, $T_{1/2}$ quinine sulfate 12−18 h, $T_{1/2}$ carbamazepine 35−40 h).

purposes prior to entry to this study. None had a neuropathy or myopathic changes on EMG.

Short Exercise Test. Short exercise tests (SETs) were performed by stimulating the ulnar nerve at the wrist and recording with surface electrodes over abductor digiti minimi, as described by Streib[18] and Fournier et al.[14] Compound muscle action potentials (CMAPs) were recorded at baseline and every 10 s during 3 short exercise trials (10-s exercise followed by 60-s rest). The amplitude changes from baseline were calculated and plotted as described previously.[15] We used amplitude-only changes, because cooling was not performed. Normative data for amplitude-only decrement at room temperature ($n = 51$, upper limit of normal 11.5%) was obtained from controls tested previously.[15]

Muscle Velocity Recordings. *Experimental Setup.* The recording technique was similar to that developed for the brachioradialis muscle,[7] but adapted to tibialis anterior (TA).[12,16] Recordings were performed on the distal third of TA, with a monopolar stimulating needle inserted perpendicularly within 1 cm of the palpated distal extent of the muscle. Stimulation currents were delivered through an insulated monopolar needle electrode (28G; TECA, Viasys Healthcare, Madison, Wisconsin) inserted to a depth of 6–8 mm, and a non-polarizable surface electrode (Kendall Q-trace; Tyco Healthcare Group, UK) placed distal and laterally on the muscle served as the anode. Rectangular pulses (0.05 ms) generated by a computer were converted to current with an isolated constant-current stimulator (DS5; Digitimer, Ltd., Welwyn Garden City, Hertfordshire, UK). Muscle activity was recorded with a concentric needle electrode (disposable 30G concentric EMG needle, Cardinal Health, Madison, Wisconsin) approximately 20 mm proximal to the stimulating needle. The ground electrode (Kendall, as above) was positioned between the stimulating and recording electrodes. Surface temperature over TA was recorded at the end of the recording.

The signal was amplified (gain 1000, bandwidth 1.6 Hz to 2 kHz) and digitized (NIDAQ-6062E; National Instruments Europe Corp., Debrecen, Hungary) using a sampling rate of 20 kHz. The electrodes were adjusted to obtain a stable negative peak response with a stimulus of 3–10 mA. Stimulation and recording were controlled by Qtrac software (© Institute of Neurology, University College London, London, UK), using the 1200RCMQ.QRP recording protocol.

Muscle Velocity Recovery Cycles at Rest. MVRCs were recorded with 1, 2, and 5 conditioning stimuli, all separated by 10-ms interstimulus intervals (ISIs). Test stimuli were delivered every 2 s. The
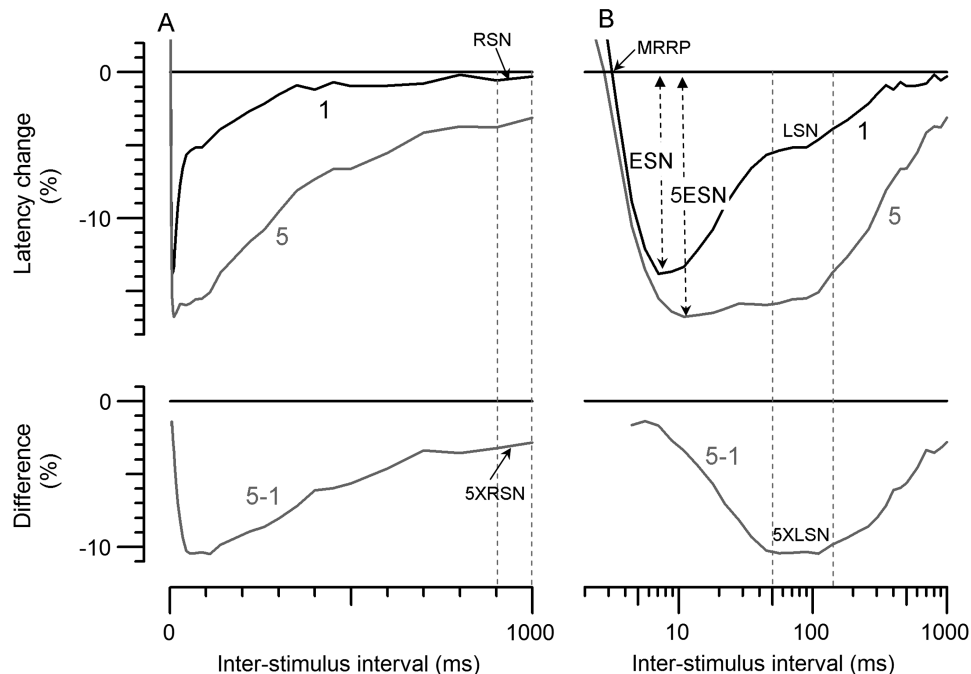
FIGURE 1. Recovery cycle measurements used for data analysis. Recovery cycles with 1 and 5 conditioning stimuli, and their difference, plotted on linear **(A)** and logarithmic **(B)** interstimulus interval (ISI) scales for a single patient with myotonia congenita. The muscle relative refractory period (MRRP), early supernormality (ESN), and late supernormality (LSN) are as defined previously, and 5ESN is the peak early supernormality after 5 conditioning stimuli. Because recovery is incomplete at 1 s in myotonia, we also defined residual supernormality (RSN) between ISIs of 900 and 1000 ms. 5XLSN and 5XRSN are the extra supernormalities after 5 conditioning impulses, taken from the difference plots. Differences between 2 and 1 conditioning pulses were calculated similarly as 2XLSN and 2XRSN (not illustrated).

ISI between the last conditioning stimulus and the test stimulus was varied from 1000 to 1.4 ms in 34 steps in an approximately geometric series (specifically 1000, 900, 800, 700, 600, 500, 450, 400, 350, 300, 260, 220, 180, 140, 110, 89, 71, 56, 45, 35, 28, 22, 18, 14, 11, 8.9, 7.1, 5.6, 4.5, 3.5, 2.8, 2.2, 1.8, and 1.4 ms).

Frequency Ramp. To characterize the effects of progressive muscle activation, the test stimulus was preceded by a 1-s train of stimuli at a frequency that was increased by 1 Hz on successive 2-s cycles from 1 to 30 Hz. The average stimulation rate was therefore increased from 0.5 to 15.5Hz over 1 min. Stimulus cycles with the test stimulus alone were recorded before (10 cycles at 0.5 Hz), during the ramp, and for a further 30 s after the end of the ramp.

Data Analysis. Recovery cycle data were analyzed by the QtracP program, as described previously.[7] The waveforms were first filtered with digital high-pass (100 Hz cut-off) and low-pass (500 Hz) filters applied both in forward and reverse time directions to provide baseline stabilization and smoothing without time displacement.[11] Response latencies were then measured from the start of the test stimulus to the negative peak of the muscle action potential. The effects of 1, 2, and 5 conditioning pulses on the latency of the test response were calculated as percentage differ-

ences compared with the latency of the test stimulus alone.

Several excitability measures were derived from the 2 recording protocols:

(a) *VRCs at rest* (Fig. 1). The MRRP was defined as the earliest (interpolated) ISI at which the latencies of the conditioned and unconditioned test responses were identical. Early supernormality (ESN) was measured as the largest percentage decrease in latency for ISIs below 15 ms. Late supernormality (LSN) was the mean percentage decrease in latency for ISIs between 50 and 150 ms. Compared with previous MVRC studies, we additionally defined "supernormality at 20 ms" (SN20) as the mean of supernormalities at 18 and 22 ms, 5ESN as the early supernormality after 5 conditioning impulses, and "residual supernormality" (RSN) as the mean percentage decrease in latency at the end of the sweep, averaged for ISIs of 900 and 1000 ms. We also defined the "extra" supernormalities 2XLSN and 2XRSN as the differences between the percentage latency decreases for 2 and 1 conditioning stimuli, and 5XLSN and 5XRSN as the differences between the percentage latency decreases for 5 and 1 conditioning stimuli.

(b) *Frequency ramp.* The measurements made during the frequency ramp are presented in Figure 2. They were the latency of the negative peak of
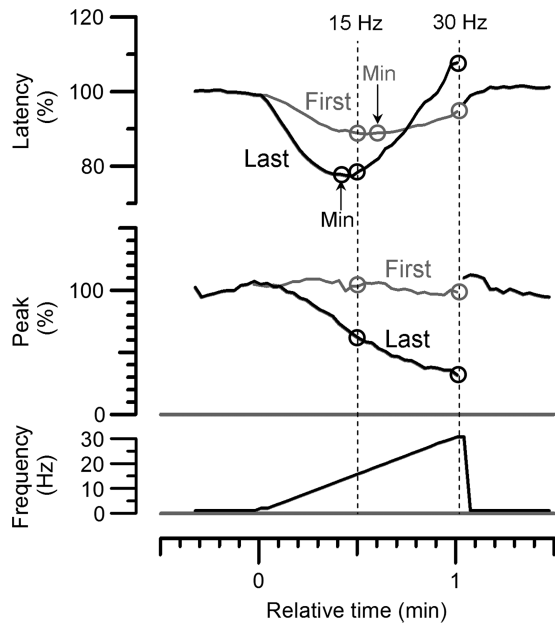
FIGURE 2. Frequency ramp measurements. Changes in latency and peak of muscle action potentials in a patient with myotonia congenita during the frequency ramp protocol, in which a 1-s train of impulses was given every 2 s. During the period of the frequency ramp, separate measurements were made of the responses to the first and last stimulus in the train. Small circles indicate points measured when intermittent stimulation was at 15 or 30 Hz, and also the frequencies when latency was minimal; for example, $Lat(15Hz)_{First}(\%) = $ percentage change in latency of first response in train, when frequency reached 15 Hz, $FLatMin_{Last}(Hz) = $ frequency when the latency to the last response in the train was minimal.

the muscle action potential, expressed as a percentage of baseline latency recorded at 15 Hz [Lat(15Hz)] and 30 Hz [Lat(30Hz)] during the ramp. Latency changes were different for the first and last responses in each 1-s train of action potentials, and these are indicated by the subscripts "First" and "Last," respectively, so that $Lat(15Hz)_{First}$ was the latency to the first of 15 conditioning pulses, expressed as a percentage of baseline. Values of $Lat(30Hz)_{Last}$ are not given, because this quantity was found to be unmeasurable in many patients when the responses became too small. Action potential peak amplitudes were recorded similarly as percentages of baseline values: $Peak(15Hz)_{First}$; $Peak(15Hz)_{Last}$; $Peak(30Hz)_{First}$; and $Peak(30Hz)_{Last}$. In all cases, the latency measurements during the frequency ramp first decreased and then increased again, describing a U-shaped trajectory, and the frequency at which the latency was minimal (determined by fitting a quadratic to each 6 adjacent points) was denoted FLatMin, again with the subscript First or Last.

Statistics. Many of the activity-dependent conduction measures failed the Lilliefors test of normality, and also the variances of several measures differed widely between groups. For intergroup comparisons we therefore applied the Welch unequal variance t-test to the ranked data,[19] and for correlations between measures we used the Spearman rho (ρ). When comparing groups with multiple t-tests or correlations, only $P < 0.01$ was considered significant, but in Tables 3 and 4, $P < 0.05$ is marked as significant for convenience in assessing individual tests.

RESULTS

Velocity recovery cycles with 2 and 5 conditioning stimuli were recorded successfully from all patients and controls, followed by the frequency ramp protocol, and (in the case of the patients only) by the short exercise test. Surface temperatures over TA were very similar between controls (mean ± SD: 30.5 ± 0.9) and patients (30.7 ± 1.0) and were not significantly different between any of the patient subgroups.

Short Exercise Test. The results of the short exercise tests at room temperature are detailed in Table 2. The short exercise test performed on the day of the muscle excitability studies was suggestive of chloride channel myotonia in 2 of 7 patients with ADMC and in 10 of 11 patients with ARMC.

Velocity Recovery Cycles. The results of the MVRCs with 1 and 5 conditioning stimuli are presented in Figures 3 and 4, and the measurements are compared in Table 3. In the unmedicated (MC_{Rx-}) patients the early increase in velocity (ESN) following a single impulse was 14% greater than in control subjects (Fig. 3A), and the increase after 5 conditioning impulses was 21% greater; these abnormalities were not seen in the patients on sodium channel blockers. Another difference between the Rx^+ and Rx^- groups was in the time at which supernormality peaked [i.e., ESN@(ms); Table 3], which was 2.4 ms later in the medicated group. The supernormality reflects the depolarizing afterpotential after an impulse, which is thought to arise because inward charge movement (mostly sodium ions) exceeds outward charge movement (mostly potassium ions) during the action potential. The membrane depolarization decays over about 1 s, as this charge on the capacitance of the sarcolemmal and t-tubule capacitance leaks away. Because chloride channels are generally considered to be responsible for most of the resting membrane conductance and because of the well-established reduction in chloride conductance in myotonia congenita, we expected the supernormality to last appreciably longer in the patients, but this was not the case (Fig. 3A). It was only when ≥2 conditioning stimuli were delivered that there was a clear prolongation of supernormality

Table 3. Velocity recovery cycle measurements compared between groups.

	NC (n = 30)	MC (n = 18)	MC_Rx− (n = 11)	MC_Rx+ (n = 7)	MC_Rx− vs. MC_Rx+
MRRP (ms)	3.50 ± 0.47	3.47 ± 0.89 $P = 0.27$ (NS)	3.17 ± 0.27 $P = 0.024$*	3.96 ± 1.30 $P = 0.51$ (NS)	$P = 0.11$ (NS)
ESN (%)	12.1 ± 2.2	13.0 ± 2.1 $P = 0.12$ (NS)	13.8 ± 1.9 $P = 0.016$*	11.8 ± 1.8 $P = 0.82^{NS}$	$P = 0.016$*
ESN@(ms)	7.7 ± 1.0	8.6 ± 1.9 $P = 0.048$*	7.7 ± 0.6 $P = 0.80$ (NS)	10.1 ± 2.3 $P = 5.2 \times 10^{-5\S}$	$P = 0.00012^{\ddagger}$
5ESN (%)	14.5 ± 2.7	15.9 ± 2.7 $P = 0.065$ (NS)	17.5 ± 1.3 $P = 4.7 \times 10^{-5\S}$	13.3 ± 2.3 $P = 0.29$ (NS)	$P = 4.5 \times 10^{-5\S}$
SN20 (%)	7.0 ± 1.4	9.2 ± 1.5 $P = 9.9 \times 10^{-7\mid}$	9.4 ± 1.7 $P = 3.8 \times 10^{-5\S}$	9.0 ± 0.9 $P = 0.00014^{\ddagger}$	$P = 0.47$ (NS)
LSN (%)	4.07 ± 0.90	4.49 ± 0.74 $P = 0.10$ (NS)	4.85 ± 0.43 $P = 0.0012^{\dagger}$	3.94 ± 0.80 $P = 0.60^{NS}$	$P = 0.020$*
RSN (%)	0.16 ± 0.24	0.19 ± 0.44 $P = 0.22$ (NS)	0.14 ± 0.45 $P = 0.75$ (NS)	0.56 ± 0.28 $P = 0.00030^{\ddagger}$	$P = 0.024$*
2XLSN (%)	2.79 ± 0.85	3.33 ± 0.60 $P = 0.0055^{\dagger}$	3.26 ± 0.56 $P = 0.033$*	3.43 ± 0.69 $P = 0.0075^{\dagger}$	$P = 0.89$ (NS)
2XRSN(%)	0.26 ± 0.20	0.64 ± 0.32 $P = 2.5 \times 10^{-6\mid}$	0.59 ± 0.23 $P = 0.00025\ddagger$	0.71 ± 0.42 $P = 5.1 \times 10^{-5\S}$	$P = 0.69$ (NS)
5XLSN (%)	8.00 ± 1.51	9.51 ± 1.27 $P = 0.00036^{\ddagger}$	10.11 ± 0.75 $P = 1.9 \times 10^{-7\mid}$	8.57 ± 1.40 $P = 0.37$ (NS)	$P = 0.010$*
5XRSN(%)	1.16 ± 0.51	2.75 ± 0.57 $P = 2.5 \times 10^{-12\mid}$	2.83 ± 0.65 $P = 6.2 \times 10^{-10\mid}$	2.63 ± 0.43 $P = 7.7 \times 10^{-11\mid}$	$P = 0.44$ (NS)

First column shows values obtained from tibialis anterior muscle in 30 normal control subjects (NC). Next 3 columns show values obtained from all 18 patients with myotonia congenita (MC), the component subgroups of 11 that were off treatment (MC_Rx−), and 7 that were on sodium channel blockers (MC_Rx+). Values given are mean ± SD, and P-value is for Welch rank test (non-parametric unequal variance t-test) for difference in median from normal controls. Last column shows P-values for comparison between MC subgroups: NS, not significant;

*P < 0.05,
†P < 0.01,
‡P < 0.001,
§P < 0.0001,
|P < 0.00001.

Table 4. Frequency ramp.

	NC (n = 30)	MC_Rx− (n = 11)	MC_Rx+ (n = 7)	MC_Rx− vs. MC_Rx+	ADMC (n = 7)	ARMC (n = 11)	ADMC vs. ARMC
Lat [15 Hz]_First (%)	93.2 ± 2.9	89.0 ± 3.5 $P = 0.00012^{\ddagger}$	92.2 ± 6.2 $P = 0.77$ (NS)	$P = 0.19$ (NS)	91.2 ± 2.6 $P = 0.11$ (NS)	89.5 ± 5.9 $P = 0.023$*	$P = 0.40$ (NS)
Lat [15 Hz]_Last (%)	83.3 ± 3.6	82.7 ± 10.8 $P=0.11$ (NS)	94.9 ± 14.0 $P = 0.014$*	$P = 0.0029^{\dagger}$	81.5 ± 5.0 $P = 0.37$ (NS)	91.3 ± 15.6 $P = 0.41$ (NS)	$P = 0.19$ (NS)
Lat [30 Hz]_First (%)	94.1 ± 3.5	93.8 ± 2.6 $P = 0.77$ (NS)	96.9 ± 4.9 $P = 0.15$ (NS)	$P = 0.92$ (NS)	93.9 ± 3.6 $P = 0.82$ (NS)	95.7 ± 4.1 $P = 0.26$ (NS)	$P = 0.25$ (NS)
FreqLatMin_First (Hz)	20.9 ± 3.4	18.1 ± 2.7 $P = 0.017$*	15.2 ± 6.3 $P = 0.043$*	$P = 0.16$ (NS)	19.3 ± 3.8 $P = 0.40$ (NS)	15.5 ± 4.6 $P = 0.00026^{\ddagger}$	$P = 0.092$ (NS)
FreqLatMin_Last (Hz)	18.7 ± 2.8	14.0 ± 2.0 $P = 5.1 \times 10^{-7\mid}$	11.2 ± 2.3 $P = 5.0 \times 10^{-10\mid}$	$P = 0.0057^{\dagger}$	14.1 ± 2.3 $P = 1.1 \times 10^{-5\S}$	12.2 ± 2.4 $P = 4.2 \times 10^{-9\mid}$	$P = 0.16$ (NS)
Peak [15 Hz]_First (%)	115.8 ± 11.2	114.2 ± 28.2 $P = 0.94$ (NS)	96.7 ± 53.0 $P = 0.66$ (NS)	$P = 0.56$ (NS)	129.7 ± 25.7 $P = 0.20$ (NS)	93.2 ± 40.6 $P = 0.17$ (NS)	$P = 0.054$ (NS)
Peak [15 Hz]_Last (%)	104.1 ± 18.6	84.2 ± 52.6 $P = 0.33$ (NS)	60.0 ± 55.9 $P = 0.26$ (NS)	$P = 0.35$ (NS)	119.2 ± 40.1 $P = 0.18$ (NS)	46.5 ± 40.9 $P = 0.00085^{\ddagger}$	$P = 0.0011$
Peak [30 Hz]_First (%)	118.3 ± 13.7	117.1 ± 25.0 $P = 0.78$ (NS)	110.2 ± 47.2 $P = 0.94$ (NS)	$P = 0.92$ (NS)	134.8 ± 32.3 $P = 0.12$ (NS)	101.5 ± 29.7 $P = 0.10$ (NS)	$P = 0.046$*
Peak [30 Hz]_Last (%)	90.2 ± 24.7	47.4 ± 35.9 $P = 0.0023^{\dagger}$	42.3 ± 51.8 $P = 0.10$ (NS)	$P = 0.47$ (NS)	77.3 ± 37.0 $P = 0.50$ (NS)	25.2 ± 30.4 $P = 1.0 \times 10^{-5\S}$	$P = 0.012$*

Effects of frequency ramp on latencies and amplitudes of muscle action potentials. The first 4 columns and figures correspond to those in Table 3. The last 3 columns show a similar comparison between 7 dominant and 11 recessive MC patients. P-values below means and SDs are for Welch rank test for difference in median values from normal controls, and P-values in last column compare MC subtypes. P-values as in Table 3.
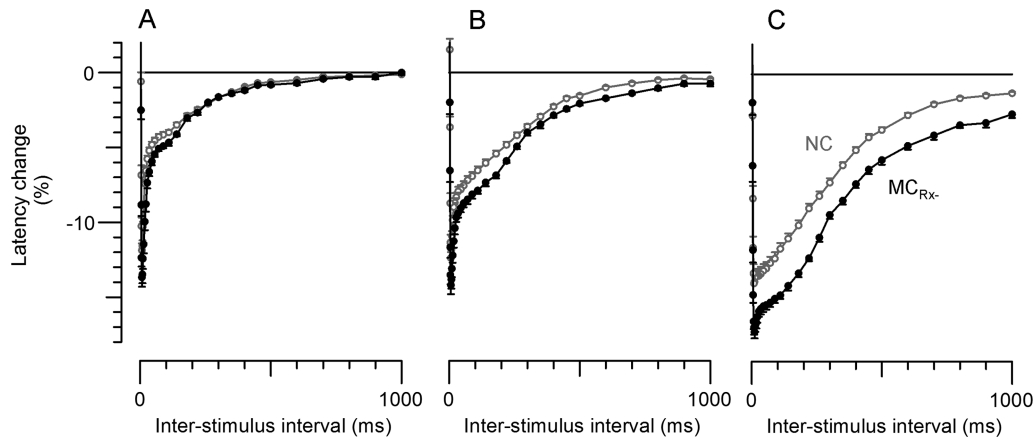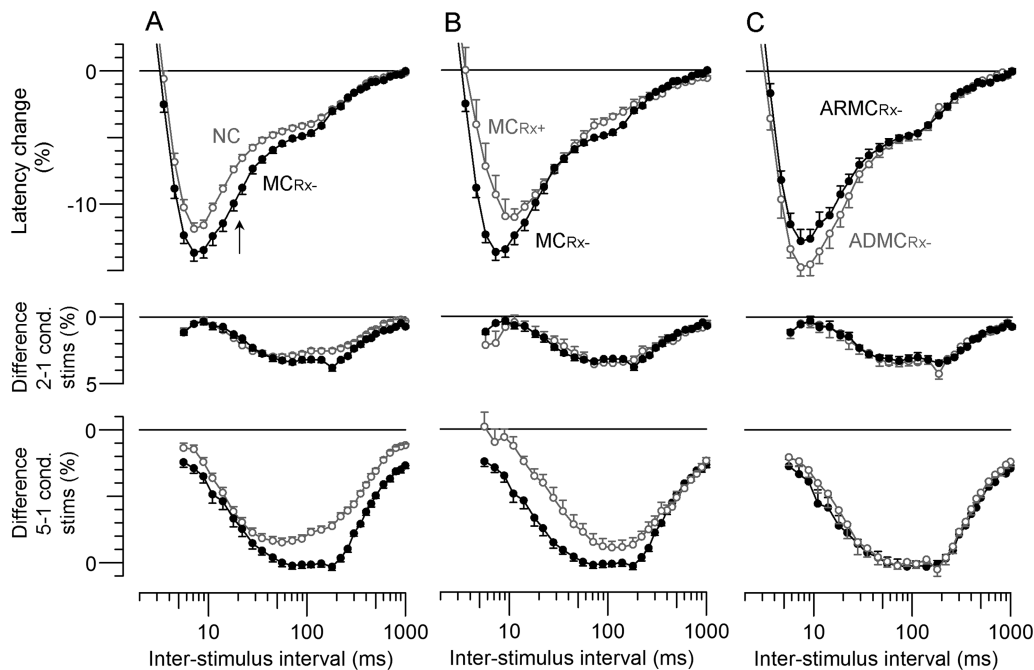
FIGURE 3. Muscle velocity recovery cycles. MVRCs for 11 myotonia congenita patients not on medication (filled black circles) and 30 normal controls (open gray circles) are compared for 1 **(A)**, 2 **(B)**, and 5 **(C)** conditioning stimuli. Data points show means with single standard error bar.

(Fig. 3B and C), and the measures of late and residual supernormality were very different between patients and controls (Table 3).

Although the recovery from depolarization occurred at a similar rate in patients and controls close to the resting potential, there was a short-lived slowing in recovery for MC patients compared with controls between about 10 and 100 ms. This is seen more clearly with a logarithmic ISI axis (Fig. 4A), so that supernormality at 20 ms (SN20: arrow) was distinctly greater in the MC patients (Table 3), whether on medication or not. Figure 4B shows that the medication only affected the earliest part of the recovery cycles. The later parts were also indistinguishable between the myotonia ADMC and ARMC subgroups (Fig. 4C). The apparent difference in peak supernormality was not significant, and in fact none of the MVRC

FIGURE 4. Muscle velocity recovery cycles of MC subgroups. **A:** MVRCs for 11 untreated MC patients (filled black circles) and 30 normal controls (open gray circles) replotted on a logarithmic timebase. Data points show means with single standard error bar. **(B)** Comparison between 11 MC_{Rx-} (black filled circles) and 7 MC_{Rx+} patients on sodium channel medication (gray open circles). **(C)** Comparison between myotonia subgroups (5 ADMC = Thomsen type; 5 ARMC = Becker type), restricted to those not on medication. Top row: percentage latency change after single conditioning impulse; middle row: additional latency change after a second conditioning impulse; bottom row: difference in percentage latency change after 5 conditioning impulses from latency change after single conditioning impulse. The arrow in **(A)** indicates ISI of 20 ms at which NC and MC latency changes diverged significantly. There were no differences between the ADMC and ARMC patients with this protocol.
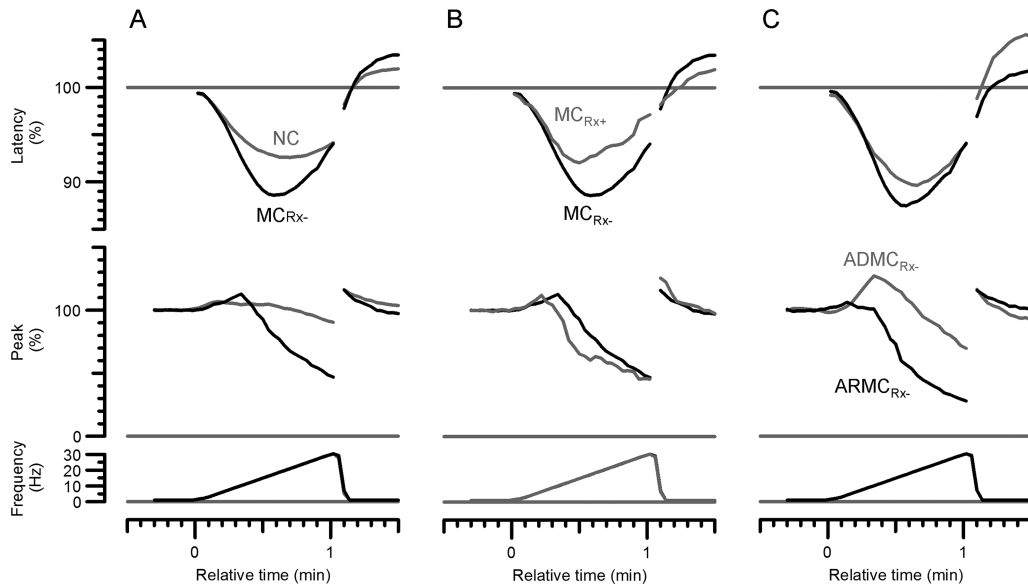
FIGURE 5. Frequency ramp. Responses of myotonia congenita patients to frequency ramp protocol, compared with those from normal controls. Muscles were stimulated for 1 s every 2 s at frequencies from 1 to 30 Hz. **(A)** Eleven MC_{Rx-} patients, plotted in black, compared with 30 normal controls, plotted in gray (means only). **(B)** Comparison between 11 MC_{Rx-} (black) and 7 MC_{Rx+} patients (gray). **(C)** Five $ARMC_{Rx-}$ patients (black) compared with 5 $ADMC_{Rx-}$ patients (gray). First row: latency as percent of control for the *first* response in each train; second row: peak amplitude as a percent of control for the *last* response in each train; third row: stimulation rate during the train.

measurements after 1–5 conditioning stimuli differed significantly between the ADMC and ARMC patients.

Frequency Ramp. The results of increasing the stimulation rate from an average of 1 Hz to 15 Hz (i.e., 30 Hz for 1 s with interval of 1 s) are illustrated in Figure 5, and measurements obtained as shown in Figure 2 are listed in Table 4. In this test, all groups exhibited a U-shaped latency curve, with initial speeding giving way to relative slowing of conduction, probably because progressive depolarization, due to potassium accumulation in the *t*-tubules, caused sodium channel inactivation. In Figure 5A the MC_{Rx-} group went "round the U" more rapidly than the controls, and the additional depolarization caused a reduction in peak amplitude, because the reduced chloride conductance was inadequate to maintain the membrane potential. (In some MC patients, the last responses in each train even became too small at the higher frequencies to measure their latency, and thus latencies to the last response in each train are omitted in Fig. 5.) The most sensitive measure to discriminate between the MC_{Rx-} and NC groups was FreqLatMin$_{Last}$ (Table 4 and Fig. 5B), the frequency at which the latency U reached a minimum for the last response in the train. This measure was reduced even more with sodium channel medication, but as with the other latency measurements it did not distinguish between the ADMC and ARMC subgroups. In contrast, the changes in peak ampli-

tude during the frequency ramp did not differ significantly between the MC_{Rx+} and MC_{Rx-} groups, but the amplitude of the last response in the train [i.e., Peak(15Hz)$_{Last}$ and Peak(30Hz)$_{Last}$] was reduced much more for ARMC than ADMC patients (Table 4). Figure 5C shows that, although the latency changes in ADMC and ARMC patients started off decreasing together, at about 10 Hz they began to diverge, and the ADMC action potentials did not drop in amplitude as much as those in the ARMC group (the early increase in amplitude in the ADMC group was not statistically significant).

Comparison with Conventional Short Exercise Test. When the amplitude decrements in the short exercise test were compared with the other measurements for the 18 myotonia patients, the strongest correlation was with Peak(30Hz)$_{Last}$%, the peak amplitude of the response to the last stimulus in the 30Hz train at the end of the frequency ramp, and the next best correlations were with Peak(15Hz)$_{Last}$% and Peak(15Hz)$_{First}$%, the amplitudes of the last and first responses in the train at 15 Hz. The values were not normally distributed, so the Spearman rho was used as a measure of correlation, which was -0.761 for Peak(30Hz)$_{Last}$% ($P = 0.00028$), -0.684 ($P = 0.0018$) for Peak(15Hz)$_{Last}$%, and -0.617 ($P = 0.0063$) for Peak(15Hz)$_{First}$%. The relationships between 2 of these measurements of activity-dependent amplitude change and SET decrement are illustrated in
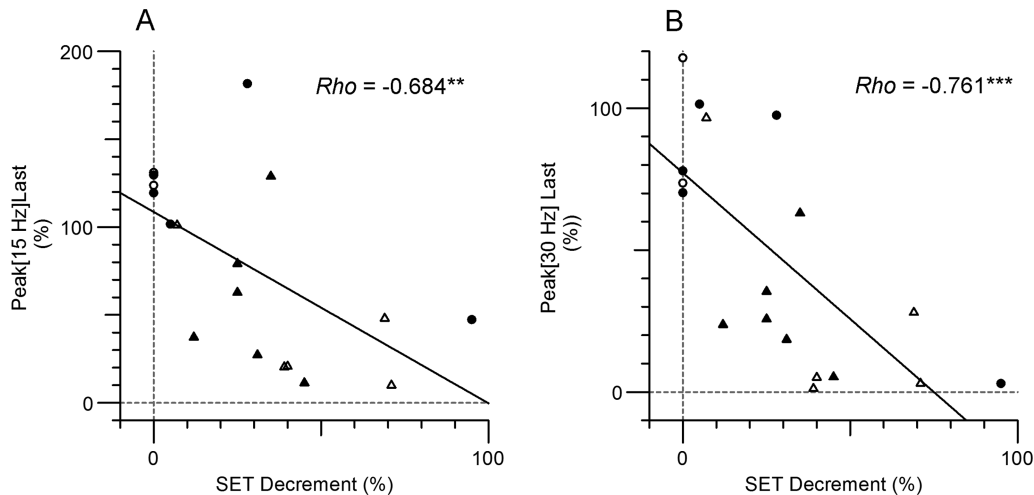
FIGURE 6. SET vs. frequency ramp. Comparison between amplitude decrement measured in the short exercise test and the changes in peak amplitude of responses during the frequency ramp: **(A)** Last response in train at 15 Hz; and **(B)** last response in train at 30 Hz. Circles = 7 ADMC patients; triangles = 11 ARMC patients; filled symbols = Rx$^-$, open symbols = Rx$^+$. Also indicated are regression lines of peak amplitudes on SET decrement and Spearman rank correlation coefficients (ρ) for all 18 patients ($^{**}P < 0.01$; $^{***}P < 0.001$).

Figure 6. The close relationship between SET decrement and the frequency ramp measures holds for patients on sodium channel blockers as well as for untreated patients. It will be noted that ARMC patients tend to have greater SET decrements and peak amplitude changes during the frequency ramp than ADMC patients, and the SET decrements and frequency ramp peak changes are the physiological measures that best distinguish ADMC from ARMC patients. However, whereas the *P*-value for comparison between ADMC and ARMC patients by the Welch rank test was 0.0022 for Peak(15Hz)$_{Last}$%, the same test applied to SET decrement did not achieve significance ($P = 0.054$).

Comparison between ADMC Patients and NC and ARMC Groups. Because the muscle abnormalities in all MC patients are attributed to dysfunctional chloride channels, and the symptoms in ARMC patients are usually more severe than those in ADMC patients, it might be expected that activity-dependent conduction changes would fall along a single line, corresponding to the percentage of available chloride channels, with ADMC intermediate between the NC and ARMC groups. One might expect that the variables in Tables 3 and 4 that best separate the NC and MC groups would be the same variables that best separate the ADMC and ARMC subgroups. Inspection of the tables, however, indicates that this is very far from the case. In Figure 7, the NC, ADMC, and ARMC group values are compared for the 3 variables that best separate MC patients from controls and also for the 2 variables that best separate the MC subgroups. In Figure 7A–C, the MVRC variables, 5XRSN and SN20,

and the frequency ramp variable, FMinLat$_{Last}$, which are the variables that differ most between the MC patients and controls, are not significantly different between the ADMC and ARMC subgroups. Similarly, in Figure 7D and E, the variables that are most different between the myotonia subgroups are not significantly different between the ADMC subgroup and controls. These comparisons suggest that the ADMC subgroup does not simply express a milder form of the chloride channel dysfunction exhibited by the ARMC subgroup but that it expresses a qualitatively distinct abnormality, as discussed below.

DISCUSSION

In this study we have used direct muscle stimulation and recording to explore whether activity-dependent conduction changes can provide useful information about the abnormal muscle membrane properties in MC. Here we have discussed the nature of the membrane changes revealed by the new tests, the insights this provides into the mechanisms underlying clinical myotonia and transient weakness in MC, and how some of these changes are ameliorated by sodium channel blockers. We have also discussed the reasoning why different membrane properties should be associated with the autosomal dominant and autosomal recessive inheritance patterns.

Muscle Membrane Properties Underlying Myotonia in MC. We found an increased early supernormality (ESN) in our untreated MC patients compared with normal controls (Table 3). The size of the ESN reflects the size of the depolarizing afterpotential (DAP) following the action potential (AP),
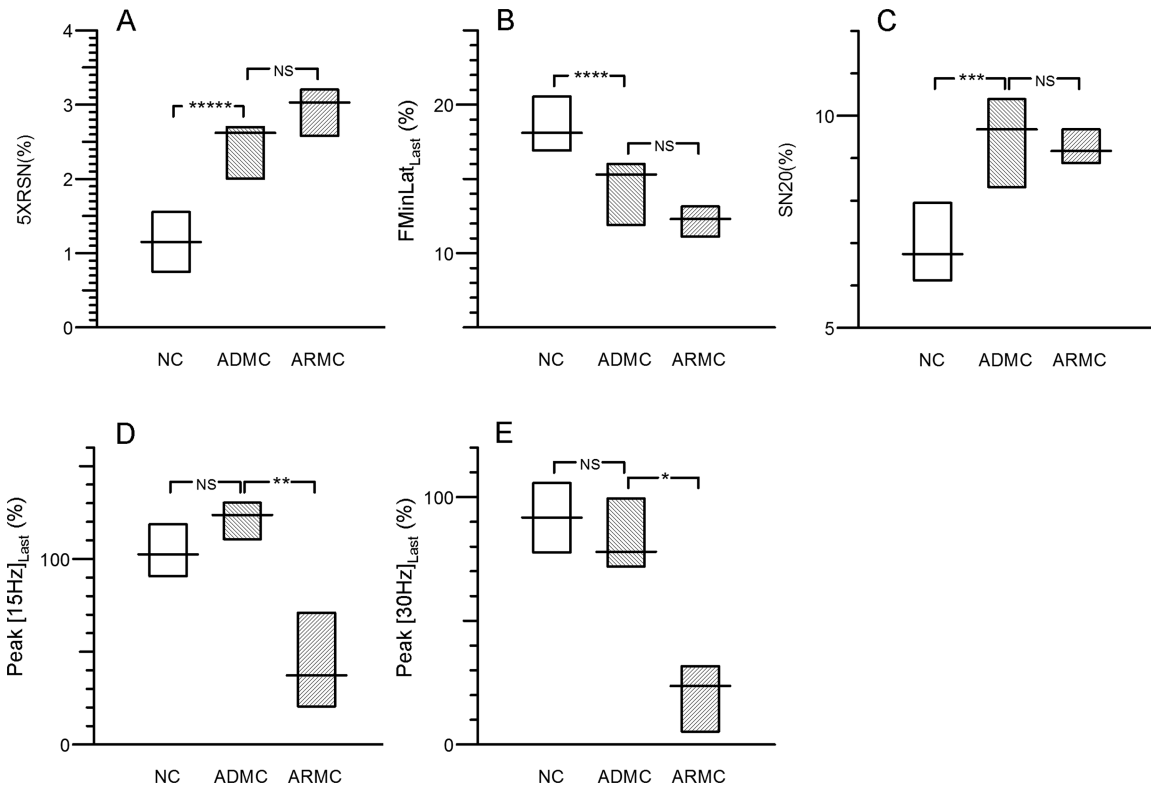
FIGURE 7. ADMC data compared with NC and ARMC. Activity-dependent conduction measures, plotted as medians and interquartile ranges, for those variables which best distinguish MC patients from normal controls **(A–C)** and for those variables which best separate ADMC and ARMC subgroups **(D–E)**. *P*-values are indicated for comparisons between adjacent groups by the Welch rank test ($^{NS}P > 0.05$, $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$, $*****P < 0.00001$). Note the overlap between the ADMC and ARMC groups in **(A)–(C)** and overlap between the NC and ADMC groups in **(D)–(E)**.

and the increased ESN in our patients is consistent with the DAP being larger due to reduced chloride conductance. In sustained myotonic discharges, the typical intraburst interval is 6.5–50 ms (intraburst frequency 20–150 Hz[20]), so it is of interest that supernormality was enhanced most significantly at 20 ms (Table 3). In addition, in MC patients, there was an increase in late supernormality (LSN), which was amplified after a train of impulses (Table 3 and Figs. 3 and 4). This late supernormality is thought to reflect the depolarizing effects of K accumulation in the t-tubules,[7] which in MC patients is larger due to loss of the normal buffering effect of chloride conductance. The recovery is also delayed after multiple conditioning stimuli, so that 5XRSN, the extra residual supernormality 1 s after 5 conditioning impulses, provides the most consistently abnormal feature of the MVRCs in MC patients (Table 3). It is likely that the cumulative late supernormality is relevant not only to facilitating repetitive firing after an AP, but also in sustaining prolonged trains of myotonic discharges. Augmentation of hyperexcitability after a rapid train of impulses may explain why clinical myotonia is more severe after a sudden strong contraction (e.g., when attempting a sprint) compared with a gentle limb movement.

Muscle Membrane Properties Underlying Transient Weakness in MC. When the loss of chloride conductance is severe, the depolarization caused by the increase in LSN after a sustained train of impulses may be sufficient to inactivate Na channels, thus leading to failure to generate APs and therefore weakness. This is illustrated by our findings during the frequency ramp, where in the ARMC patients, a rapid train of impulses (≥ 15 Hz) was associated with a fall in amplitude of the compound muscle fiber response, analogous to the fall in CMAP in the short exercise test. This is discussed in more detail later when comparing ADMC with ARMC.

How Does Medication with Sodium Channel Blockers Reduce Myotonia in MC?. We were unable to test the same patients on and off drug, so we can only make limited inferences about the effects of medication. Nevertheless, our recordings provide some insights into how sodium channel blockers reduce myotonic discharges in patients with MC. Figure 4B indicates that the principal effects of Na channel blockade were to reduce the peak supernormality and delay its onset, and this reduction was more pronounced after a train of 5 impulses. In 6 of 7 cases, the Na channel blocker was mexiletine,

whereas the patient on carbamazepine and quinine exhibited qualitatively similar differences from the drug-free group. The results suggest that the blockade results in delayed and reduced opening of Na channels during the AP, causing delayed and reduced DAP and associated supernormality. These observations are consistent with the effects of mexiletine on wild-type Nav1.4 channels *in vitro*[21] and provide some insights into how Na channel blockade may alleviate myotonia in MC patients, because the effect on ESN is counter to that of reduced chloride conductance. In addition, as Na channel blockade did not appear to affect the increase in late supernormality after a train of impulses (5XLSN and 5XRSN; Table 3), this may explain why Na channel blockers are only partially effective in relieving myotonia in patients with MC. Na channel blockade also did not appear to affect peak amplitudes, so that the differences between the ADMC and ARMC subgroups in the bottom part of Table 4 were similar if only untreated patients were averaged (Fig. 5C).

MVRCs and Resting Chloride Conductance. It is often stated that chloride channels are responsible for 80–85% of the resting membrane conductance of skeletal muscle fibers.[1,2,22,23] Assuming that chloride conductance is reduced by at least half in MC patients (since heterozygous carriers of recessive mutations are asymptomatic), the figure of 80% implies that resting membrane conductance should be reduced by at least 40% and membrane time constant increased by at least 60% in the patients. The results in Figure 3A, however, show no such evidence. Decay of supernormality after a single impulse, presumably reflecting decay of the depolarizing afterpotential, was very similar to normal. Only after 2 or 5 impulses was the decay to baseline notably slowed (Fig. 4B and C). Further light was shed on this unexpected behavior by Figure 4A, in which it is seen that for a period up to about 100 ms after a single impulse, the supernormality was distinctly greater in patients with MC, a difference that was highly significant at 20 ms (Table 3). This suggests that the chloride channels only played a major role in normalizing the membrane potential for a limited period after the action potential, whereas the membrane was still depolarized. Consistent with this, when the supernormality was supplemented by the prominent late afterpotential after 5 conditioning impulses, as potassium ions accumulated in the t-tubule system, the chloride channels were clearly active again in the normal subjects, because, in the patients, the late afterpotential was relatively enhanced and prolonged.

The explanation for this unexpected behavior is that resting chloride conductance is much lower than once thought. The figure of 85% of resting membrane conductance was obtained from *in vitro* preparations, in which the resting potentials measured with sharp electrodes averaged about −75 mV,[24,25] whereas *in vivo* recordings from human muscle fibers showed a normal resting potential of −91 mV in a previous study.[26] This discrepancy in resting potentials is important, because muscle chloride channels are voltage-dependent; they deactivate on hyperpolarization and activate on depolarization.[27] Furthermore, intracellular adenosine triphosphate (ATP) levels were not controlled in the *in vitro* experiments, and the high level of ATP in resting muscle effectively inhibits CLC-1 activity by shifting voltage gating to more positive potentials.[28] A recent *in vitro* study with internal ATP controlled, showed that resting muscle chloride conductance was just under 10% of maximum at −90 mV and increased steeply with membrane depolarization, with a time constant of about 10 ms.[29] The recordings in Figures 3 and 4 support this model, in which chloride channel conductance is quite low in the resting state, but increases during the early part of the DAP (e.g., at 20 ms), with a time lag because of the slow kinetics. After 5 action potentials the cumulative late afterpotential becomes large enough to provide prolonged chloride channel activation, the lack of which is evident in MC patients. Therefore, the CLC-1 chloride channels should not be thought of as simple leakage channels, but as channels that act specifically to limit membrane depolarization.

Repetitive Stimulation and Separation of ADMC from ARMC. It has long been recognized that some MC patients have a decrementing CMAP response to repetitive stimulation, which correlates with presence of transient weakness rather than with the severity of myotonia[30–33] A large decrement in response to 10-Hz repetitive stimulation is generally thought to be characteristic of ARMC, similar to the large decrements in the short exercise test, although there are exceptions.[34] Accordingly, in our frequency ramp recordings, there was a separation of the MC subtypes. This was seen most clearly in the decline in peak amplitude for the last response in each train of the frequency ramp, which was much more pronounced for the ARMC group, both at 15 and 30 Hz (Table 4 and Fig. 5). As suggested above, the decline in peak amplitude presumably occurred because there was insufficient time between trains to clear the potassium accumulation in the t-tubules, resulting in a progressive membrane depolarization and resultant inactivation of sodium channels. This potassium-driven membrane depolarization is normally limited by activation of the high chloride conductance, so the

observation that the action potentials dropped in amplitude during the ramp much more for the ARMC patients than the ADMC patients suggests that, in this situation, effective chloride conductance was lower in the former group.

Why Should Chloride Channels Behave Differently in Myotonia Subgroups?. Our recordings indicate that ADMC and ARMC muscles behave similarly when depolarized by up to 5 stimuli, but that ARMC muscles become more depolarized during long trains of impulses. The implication that chloride conductance is increased by depolarization in ADMC muscles is consistent with current ideas about the structure of chloride channels and their mutations in MC. The CLC-1 channels are now known to be dimers with 2 pores which are controlled by 2 gates in series: a "common gate," which affects current through both pores, and 2 independent "fast gates," which each control current through a single pore.[23] ADMC is associated with mutations affecting the common gate, usually by shifting its voltage dependence so that more depolarization than normal is needed to open the channel.[35–37] In ARMC, on the other hand, the mutations usually affect only a fast gate, but with both alleles affected total loss of function may result.[23]

There are exceptions to these generalizations, because the same mutation can apparently cause ADMC in one family and ARMC in another,[38] but they provide a simple explanation for our findings. In ADMC and ARMC, chloride conductance at rest and on mild depolarization can be similarly reduced, resulting in the comparable MVRC abnormalities shown in Figure 7A–C. However, when subjected to long trains of impulses and progressive membrane depolarization due to potassium accumulation, the common gates of the chloride channels in ADMC patients are opened, thus limiting the extent of membrane depolarization. Whereas the depolarization in ARMC patients causes reduced muscle action potentials and can be sufficient to reduce calcium release and cause weakness, in ADMC the depolarization is self-limiting, and there is no weakness.

In conclusion, we have found that MVRCs provide evidence of an enhanced depolarizing afterpotential in MC patients, which may help trigger myotonic discharges, but it is counteracted by sodium channel blockers. The MVRCs contradict the idea that CLC-1 channels provide the major component of resting membrane conductance; depolarization is required to activate the CLC-1 channels and to reveal the reduced chloride conductance in MC patients. The ADMC and ARMC subgroups had very similar MVRCs, but the decline

in amplitude during the frequency ramp, which correlates with the amplitude decrement in the short exercise test, was much greater in the ARMC than in the ADMC patients. This is most likely because the CLC-1 mutation in ADMC does not totally inactivate the channels but only shifts the voltage dependence of the common gate, so that the channels become activated when depolarized, and depolarization is self-limiting.

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