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Abbreviations

CMAP: compound muscle action potential

DM1: Myotonic dystrophy type 1

DM2: Myotonic dystrophy type 2

DMPK: Dystrophia myotonica – Protein kinase

ESN: early supernormality

ISI: inter-stimulus interval

LSN: late supernormality

MC: myotonia congenita

MRRP: muscle relative refractory period

MSN: mean supernormality

MVRC: muscle velocity recovery cycle

RSN: residual supernormality

SET: short exercise test

SN20(%): Supernormality at 20ms (mean of supernormalities at 18 and 22 ms)

TA: tibialis anterior

5ESN: early supernormality after 5 conditioning stimuli

5SN20(%): Supernormality at 20ms after 5 conditional stimuli.

5XLSN: extra late supernormality after 5 conditioning pulses (similarly, 5XRSN).

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ABSTRACT *(Max 150 words)*

Introduction: Myotonia in myotonic dystrophy types 1 (DM1) and 2 (DM2) is generally attributed to reduced chloride channel conductance. We used muscle velocity recovery cycles (MVRCs) to investigate muscle membrane properties in DM1 and DM2, with comparisons with myotonia congenita (MC).

Methods: MVRCs and responses to repetitive stimulation were compared between patients with DM1 (n=18), DM2 (n=5), MC (n=18), and normal controls (n=20).

Results: Both DM1 and DM2 showed enhanced late supernormality after multiple conditioning stimuli, indicating delayed repolarization as in MC. Contrary to MC, however, DM1 showed reduced early supernormality after multiple conditioning stimuli, and weak DM1 patients also showed abnormally slow latency recovery after repetitive stimulation.

Discussion: These findings support impaired chloride conductance in both DM1 and DM2. The early supernormality changes indicate that sodium currents were reduced in DM1, while the weakness-associated slow recovery after repetitive stimulation may provide an indication of reduced Na^+/K^+-ATPase activation.

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INTRODUCTION

Myotonic dystrophy (DM1) is the most common muscular dystrophy in adults. It is caused by the expansion of an unstable trinucleotide $(CTG)_n$ repeat in an untranslated, but transcribed, portion of the 3' region of the DM1 protein kinase (*DMPK*) gene on chromosome 19q13.3[1] .

The main mechanisms currently thought to underlie the multisystemic abnormalities in DM1 can be divided into: (a) a gain-of-function effect leading to RNA toxicity due to the transcribed mutant *DMPK* mRNA affecting RNA splicing factors, resulting in abnormal splicing of mRNA transcripts of various proteins [including the muscle chloride ion channel (CLC-1)]; (b) altered expression of neighboring genes such as *SIX5* (causing cataracts); and (c) abnormalities in the structure, enzymatic activity, and subcellular localization of the DMPK protein itself [2-8].

In DM1, the myotonia is thought to arise due to misregulation of alternative splicing of the muscle chloride channel CLC-1[9,10], together with transcriptional downregulation of *CLCN1* due to leaching of the transcription factor SP1 by mutant RNA[11]. Less emphasis is given to the early suggestions that sodium channel dysfunction may be relevant to myotonia in DM1[2,3].

DMPK is a serine/threonine kinase and has been shown to modulate skeletal muscle Na^+ channels[3,12] and Ca^{2+} homeostasis[4]. Although there have been contradictory results about the effects of the DM1 mutation on the levels of DMPK mRNA and protein levels in patient tissues[13-16], typically, muscle fibers and/or cultured skeletal muscle cells of DM patients exhibit a decreased resting membrane

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potential[17-19], and increased basal cytosolic Na^+ and Ca^{2+} concentrations[20-22], which are likely to be relevant to the muscle pathophysiology in DM1.

Myotonic dystrophy type 2 (DM2), initially termed proximal myotonic myopathy, is caused by an unstable tetranucleotide $(CCTG)_n$ repeat expansion in the first intron of the cellular nucleic acid-binding protein (*CNBP*) gene on chromosome 3q21.3 [23,24]. Although patients with DM2 have some clinical features in common with DM1 (such as myotonia, cataracts, and diabetes), there are significant differences, in that life expectancy is typically normal in DM2, muscle weakness is usually of late onset (50-70 years), there is no congenital form, muscle atrophy is rarely seen, and bulbar and respiratory weakness is exceptional[25]. As in DM1, the disruption in cellular function due to the pathogenic effects of the $(CCUG)_n$ RNA expansion are thought to underlie the multisystemic features in DM2, although there is evidence that *CNBP* haploinsufficiency in itself may account for many of the changes in skeletal and cardiac muscle. This includes the myotonia in DM2, which appears to be due to downregulation of CLC-1, secondary to low levels of *CNPB*, without mis-splicing of CLCN1 [26].

We have recently used muscle velocity recovery cycles (MVRCs) and a repetitive stimulation protocol to investigate how membrane function is affected in patients with myotonia congenita (MC), and found evidence of an enhanced depolarizing afterpotential, which was reduced in patients treated with Na^+ channel blockers. In addition, contrary to the widely held view that chloride conductance makes a large contribution to resting membrane conductance, we found that chloride conductance only became important when muscle fibers were depolarized[27].

In this study, we used the same protocol to investigate how membrane function is altered in DM1 and DM2 patients, with the aim of confirming the expected

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reduction of chloride channel conductance, and to investigate whether there was evidence of concomitant sodium channel dysfunction in the DM1 patients. We compared the findings to those of MC patients, and to age-matched normal controls.

METHODS

Patients

All 18 DM1 patients and 5 DM2 patients were genetically confirmed. The patients were aged 43.9 ± 15.1 years (mean ± SD), range 20-73 years (Table 1). There were 9 men and 14 women. Three patients (2 DM1, 1 DM2) were diabetic. Eighteen previously studied[27] patients with genetically confirmed myotonia congenita (11 ARMC, 7 ADMC) were included for comparison. Eleven of these patients (6 ARMC, 5 ADMC) were designated as 'Rx-' (i.e. these patients were either taking no medication for myotonia, or had omitted the medication for >5 times the half-life of the drug at the time of the study). Another 7 MC patients (5 ARMC, 2 ADMC), were taking mexiletine or carbamazepine and were designated 'Rx+'.

Asymptomatic Controls

The MVRC studies were compared with recordings from 20 healthy volunteers, 5 men, 15 women, aged 44.1 ± 13.3 years (range 27-69) who served as normal controls (NC).

Consent

Informed written consent was obtained from all patients and controls according to the Declaration of Helsinki. This study was approved by the St Thomas' Hospital Research Ethics Committee, London, UK.

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Study Protocol

All the patients had standard nerve conduction studies, muscle velocity recovery studies, the short exercise test at room temperature, and a blood sample for electrolytes and glucose taken on the same day (within 2 hours of the studies). Ankle dorsiflexion power was graded (MRC scale) on the day of the study.

Short Exercise Test

Short exercise tests (SETs) were performed by stimulating the ulnar nerve at the wrist and recording with surface electrodes on abductor digiti minimi. Compound muscle action potentials (CMAPs) were recorded at baseline and every 10s during 3 short exercise trials (10s exercise followed by 60s rest). The amplitude changes from baseline were calculated and plotted as described previously [28].

Muscle velocity recordings

Experimental setup

The recording technique was as described previously for tibialis anterior (TA)[29,30,27]. Recordings were performed on the distal third of TA, with the 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, USA) inserted to a depth of 6-8 mm, while 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 by means of a concentric needle

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electrode (disposable 30G concentric EMG needle, Cardinal Health, Madison, Wisconsin, USA) approximately 20 mm proximal to the stimulating needle. The ground electrode (Kendall, as above) was positioned between the stimulating and recording electrodes. Patients were seen in a heated room and kept warm in an effort to achieve a skin temperature as near as possible to 32 deg C at the start of the study. Surface temperature over TA was recorded at the end of the recording.

The signal was amplified (gain 1000, bandwidth 0.2 Hz to 3 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 (written by H. Bostock, Institute of Neurology, London, UK), using the 1200RCMQ.QRP recording protocol.

Muscle Velocity recovery cycles (MVRCs) at rest

MVRCs were recorded with 1, 2, and 5 conditioning stimuli, all separated by 10 ms inter-stimulus intervals (ISI). Test stimuli were delivered every 2s. The 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, delivery of 1 stimulus every 2 sec was replaced by a 1-sec train of stimuli delivered every 2 sec, with the number of stimuli in the train increased by 1 from 2 to 30 in successive 2-sec cycles (see Fig. 4 in Boerio et al., 2012)[29]. During the frequency ramp, the mean

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stimulation rate increased from 1 to 15 Hz over 1 minute, and responses were measured to the first and last stimuli in each train. Stimulus cycles with the test stimulus alone were recorded before the frequency ramp (10 cycles at 0.5Hz) and for 30 sec after the end of the ramp.

Data analysis

Recovery cycle data were analyzed by the QtracP program, as previously described[31]. 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[32]. 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 differences compared to the test stimulus alone.

Several excitability measures were derived from the 3 recording protocols:

a) VRCs at rest. 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. We 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 5XLSN and 5XRSN as the differences between the percentage

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latency decreases for 5 and 1 conditioning stimuli. Compared with previous MVRC studies, we additionally defined 'supernormality to 5 conditioning stimuli at 20ms' (5SN20) as the mean of supernormalities at 18 and 22ms to 5 conditioning stimuli.

b) Frequency ramp. From the frequency ramp recordings we measured the latency of the negative peak of the muscle action potential, expressed as a percentage of baseline latency, at 15Hz [Lat(15Hz)] and 30Hz [Lat(30Hz)] during the ramp, and 30s after the end of the ramp Lat(30Hz+30s)%. During the ramp, responses to the first and last stimuli in the train were designated with the subscript $_{First}$ or $_{Last}$ respectively.

Statistics

Many of the activity-dependent conduction measures failed the Lilliefors test of normality, and because of the small sample size of the groups, for intergroup comparisons we used the Mann Whitney U test, and for correlations between measures we used the Spearman rho (ρ). When comparing groups with multiple U-tests or correlations, only $P<0.01$ was considered significant, but for discussion, $P<0.05$ is mentioned when relevant for individual tests.

RESULTS

MRC grading of Tibialis Anterior

The strength of the relevant tibialis anterior muscle was graded (MRC scale) prior to the muscle excitability studies, and the results are shown in Table 1. For the purposes of comparing excitability data between weak and strong muscles, MRC

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grades below 4 were classified as 'weak', and MRC 4-5 were classified as 'strong'. For correlation studies, MRC 4- was designated as 3.5 and 4+ as 4.5.

Nerve conduction studies

One non-diabetic DM1 patient had an asymptomatic mild sensory neuropathy. None of the 3 diabetic patients had neuropathy.

Short exercise test

The results of the SETs at room temperature are detailed in Table 1. The SET performed on the day of the muscle excitability studies was suggestive of chloride channel myotonia in 13 DM1 patients and 1 DM2 patient, and showed a normal pattern without significant decrement in the other patients. It was omitted in 1 DM1 patient.

Velocity recovery cycles

The results of the MVRCs with 1 and 5 conditioning stimuli are illustrated in Figure 1A and B, respectively, and the measurements are compared in Table 2.

The overlap of the MVRCs following single stimuli in Fig. 1A suggest that resting potentials are similar in the 3 groups, and this is supported by the similar values of relative refractory period (MRRP) in Table 2. MRRP is affected by temperature[33], so it is important to note that skin temperatures overlying the muscle were closely matched between groups (NC: 29.9°C ± 1.2; DM1: 30.0 ± 1.3; DM2: 30.4 ± 0.6).

In both DM1 and DM2 patients, there was an increase in the residual supernormality to multiple conditioning stimuli, similar to that observed in patients

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with myotonia congenita (MC) [27], and attributable to impaired chloride conductance. Fig 1D shows that this highly significant abnormality was present equally in the stronger and weaker DM1 patients.

Although the later parts of the recovery cycles after 5 conditioning impulses were very similar in DM1 and DM2 patients (Fig. 1B) and were qualitatively similar to the MC patients, the earlier parts diverged. At 20 ms, the MC patients showed a substantial increase in supernormality compared with controls, the DM2 patients showed only a trend in this direction, and the DM1 patients showed a significant decrease (Table 2). Fig. 1C shows that this contrasting behavior to the MC patients was primarily attributable to the weaker DM1 patients.

Fig. 2 shows more clearly the separation of DM1 and MC patients and normal controls by the 2 MVRC measures used in Fig. 1C and D. The possibility that the difference between DM1 and MC patients may relate to sodium channel availability is suggested by the comparison with the MC patients taking sodium channel blockers, as against those not on treatment.

Frequency Ramp

The effects of increasing the stimulation rate from an average of 1 Hz to 15 Hz (i.e. 30 Hz for 1s with an interval of 1s) for patients and controls are listed in Table 3. The responses of the weaker DM1 patients to the frequency ramp, illustrated in Fig. 3, differed from all the other groups. The responses to the first stimulus in each 1 sec train (i.e. after a 1 sec recovery period from the last train) did not differ significantly in latency from the controls during the frequency ramp. After the end of the frequency ramp, however, when the stimulation rate had reverted to once every 2 sec, the latencies of the weaker DM1 patients took much longer to recover (Fig. 3B). The time taken for the mean latency to recover to its value before the ramp was 8.4s in the

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controls, but 47s in the weak DM1 group. Fig. 3D shows that, of all the groups tested, only the latencies of the weaker DM1 patients had failed to recover 30 sec after the end of the ramp. Fig. 3E shows how this measurement was strongly related to strength, as assessed by MRC grade. In DM1 patients, strength was negatively correlated with age ($\rho = -0.63$, $P=0.0052$), but not as strongly as with Lat(30Hz+30s) ($\rho = 0.75$, $P = 0.00038$). There was no significant correlation between MRC grade and decrement on the short exercise test ($\rho = 0.061$, $P = 0.80$), or with the CTG expansion size ($\rho = 0.25$, $P = 0.56$).

DISCUSSION

In this study we used a direct muscle stimulation paradigm to investigate the muscle membrane properties in patients with DM1 and DM2, and compared the findings against normal controls and with those we obtained previously using the same stimulation protocol in MC patients. Here we discuss the similarities and differences between the groups, and the insights they give into chloride channels and sodium transport in the DM1 and DM2 patient groups.

Comparisons between MVRC recordings in DM1, DM2, and MC patients

Changes in residual supernormality attributed to reduced chloride conductance.

The most striking and consistent abnormality in the recovery cycles of the DM patients was the slow return to baseline latency after 5 conditioning stimuli (Fig. 1B), which was assessed by the residual supernormality, 950 ms after 5 conditioning stimuli (5XRSN) (Fig. 1D, Table 3). This was very similar to our finding in MC patients[27] and presumably reflects the well-established reduction in chloride conductance in DM1[9,11] and DM2[26]. As in the case of the MC patients, the reduced

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chloride conductance had little effect on the recovery cycle following a single conditioning impulse (Fig. 1A), because chloride channels only make a major contribution to muscle membrane conductance when the fibers become depolarized.

Significance of changes in early supernormality.

Although the late components of the recovery cycles are similar for DM1, DM2, and MC patients, the early parts show clear differences. While the DM2 patients resembled the MC patients in having an increased early supernormality (SN20, 5SN20) compared with controls, this was not the case with the DM1 patients who, by contrast, showed a reduced early supernormality (Fig. 1B), mostly due to the weaker DM1 patients (Fig. 1C, Table 3). In the MC patients, the increased early supernormality was attributed to an increased depolarizing afterpotential related to the reduced chloride conductance[27]. In the DM2 patients, the tendency towards increased early supernormality was therefore consistent with the reduction in chloride conductance. So what was the explanation for the opposite change in early supernormality seen in the weak DM1 patients? Apart from the chloride conductance, the amplitude of the depolarizing afterpotential and related supernormality depends on the balance between inward sodium and outward potassium charge movements during the action potential, and there are 2 reasons why sodium channel currents may be reduced.

One possibility, suggested by comparison with the MC patients taking sodium channel blockers (Fig. 2), is that sodium channel availability was reduced in the DM1 patients. In this case medication could not be responsible, since only 1 of our DM1 patients (and none of the weaker ones) was taking a sodium channel blocker (Table 1). It has been known for some time that DMPK itself has a role in modulating skeletal sodium channels. In 2000, Mounsey et al[12] described a 50% reduction in peak

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Na^+ current amplitude in both *DMPK*$^{-/-}$ and *DMPK* $^{+/-}$ mouse myocytes[12] which is not due to a reduction in Na^+ channel expression but is thought to be related to silencing of muscle sodium channels[34]. A second possibility is that a deficiency in sodium pumping results in both depolarization of the resting membrane potential and a raised intracellular sodium concentration ($[Na^+]_i$) (see below), both of which would act to reduce inward sodium currents during the action potential. Membrane depolarization would normally be expected to prolong muscle relative refractory period, but a reduction in sodium currents due to a reduced sodium gradient may have a contrary effect, so that our observation that MRRP was not significantly prolonged in DM1 (Table 2) does not rule out this second possibility.

Frequency Ramp changes and the sodium pump

In patients as in normal controls, latencies decreased progressively as stimulation rate was increased and then started to increase again (Fig. 3), probably because the progressive depolarization due to potassium accumulation in the t-tubules inactivates sodium channels. At the end of the frequency ramp, when the stimulation rate returned to 0.5 Hz, the latency of the controls recovered within a few seconds to its pre-ramp value, and then overshot the mark and continued at a slightly higher level for the rest of the recording. The rapid recovery and overshoot are probably due to activation and sensitization of the sodium pump to $[Na^+]_i$ by the impulse train. Buchanan et al (2002)[35] found that in rat skeletal muscle, sodium pump activity is increased up to 20-fold by short trains of impulses (e.g. 60 Hz for 10s), so that after a brief increase $[Na^+]_i$ is actually reduced. In contrast, when $[Na^+]_i$ was increased by electroporation or the ionophore monensin, sodium pump activity was increased much less, and $[Na^+]_i$ recovered more slowly and without undershoot. The rapid pump

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response to impulse trains is apparently driven not by the increase in $[Na^+]_i$, but by release from sensory nerve endings of CGRP, which via cAMP, protein kinase A, and a small auxiliary protein phospholemman, causes an increase in the affinity of the Na^+/K^+-ATPase for $[Na^+]_i$ [35,36]. The much slower than normal recovery from the frequency ramp in the weak DM1 patients (Fig. 3B), indicates that the pump is not activated in the normal way, perhaps because of a deficiency in this rapid response mechanism. A weaker response of the sodium pump to muscle activation in DM1 was previously indicated by a much greater than normal release of potassium ions into the circulation following a brief period of exercise [37]. Perhaps also relevant to our finding of a strong correlation between latency recovery after the frequency ramp and muscle strength (Fig. 3E: $\rho = 0.75$, $P < 0.001$), a similarly strong correlation between muscle strength and ^{3}H-ouabain binding sites has been found in biopsied vastus lateralis from DM1 patients ($r = 0.60$, $P < 0.001$)[38]. It therefore seems likely that latency recovery after the frequency ramp provides an index of sodium pump dysfunction in DM1 and that our results reinforce the evidence of an association between weakness in DM1 and sodium pump dysfunction.

A correlation between weakness and sodium pump dysfunction could occur: (a) because the weakness results in downregulation of the pump; (b) because the pump dysfunction causes weakness; or (c) because both are caused by a third factor. There is evidence for both (a) and (b), which may mutually reinforce each other. Clausen[39] reviewed the abundant evidence that training leads to an upregulation and inactivity to a downregulation of muscle sodium pumps, and the correlation between muscle sodium pump content and muscle strength found in DM1 patients by Andersen et al.[38] was attributed to inactivity. On the other hand, weakness due to amyotrophic lateral sclerosis and peripheral neuropathy was associated with an

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increase, rather than a decrease in pump sites[40], and the abnormal release of potassium into the circulation after brief exercise was peculiar to myotonic dystrophy[37]. This likely indication of pump dysfunction was not found in 88 patients with a variety of neuromuscular disorders, nor in 4 patients with limb girdle or Becker muscular dystrophy. A plausible reason why the pump response to exercise should be reduced in DM1, but not in DM2 or other neuromuscular disorders, is that phospholemman is a substrate for DMPK[41] and phospholemman is involved in the acute regulation of the Na^+/K^+-ATPase response to exercise in human skeletal muscle [42].

DM1 is a progressive multi-systemic disorder that resembles in many respects premature aging[43]. The weakness is associated with muscle atrophy and cannot be accounted for simply by sodium pump dysfunction, leading us to option (c) above, that the correlation is due to a common causal factor. The progressive nature of DM1 is most likely explained by the fact that the CTG repeat number is not stable but tends to increase throughout life and to increase differently in different tissues[44,45]. The mechanism of CTG repeat expansion appears to be due to misguided attempts by DNA repair mechanisms to correct inappropriate conformations due to an existing repeat. The paradoxical consequence of this is that cells with the most active DNA repair mechanisms, such as muscle stem cells (satellite cells) are the most prone to expand their repeats until they can no longer support regeneration, and muscle degeneration ensues[43] . The correlation we observed between weakness and slow recovery from the frequency ramp (Fig. 3) may therefore simply reflect a common dependence on the progressive increase in CTG repeat number with age. CTG repeat number correlates inversely with DMPK mRNA and protein[13] , and DMPK deficiency

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is expected, via phospholemman, to reduce the rapid response of the sodium pump to muscle activation.

In conclusion, we have found that MVRCs in DM1 and DM2 patients provide evidence of a delayed repolarization after short trains of muscle action potentials, similar to that seen in MC patients, and attributable to a reduction in chloride conductance. The slow recovery after the 1-minute frequency ramp, which was strongly related to weakness in the DM1 patients, is most likely an indication of a reduced ability of the sodium pump to restore ionic gradients in these patients.

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REFERENCES

1. Brook JD, McCurrach ME, Harley HG, Buckler AJ, Church D, Aburatani H, , et al. Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member. Cell 1992;69(2):385.

2. Franke C, Hatt H, Iaizzo PA, Lehmann-Horn F. Characteristics of Na+ channels and Cl- conductance in resealed muscle fiber segments from patients with myotonic dystrophy. J Physiol 1990;425:391-405.

3. Mounsey JP, Xu P, John JE, 3rd, Horne LT, Gilbert J, Roses AD,et al. Modulation of skeletal muscle sodium channels by human myotonin protein kinase. J Clin Invest 1995;95(5):2379-2384.

4. Benders AA, Groenen PJ, Oerlemans FT, Veerkamp JH, Wieringa B. Myotonic dystrophy protein kinase is involved in the modulation of the Ca2+ homeostasis in skeletal muscle cells. J Clin Invest 1997;100(6):1440-1447.

5. Lee HC, Patel MK, Mistry DJ, Wang Q, Reddy S, Moorman JR, et al . Abnormal Na channel gating in murine cardiac myocytes deficient in myotonic dystrophy protein kinase. Physiol Genomics 2003;12(2):147-157.

6. Wansink DG, van Herpen RE, Coerwinkel-Driessen MM, Groenen PJ, Hemmings BA, Wieringa B. Alternative splicing controls myotonic dystrophy protein kinase structure, enzymatic activity, and subcellular localization. Mol Cell Biol 2003;23(16):5489-5501.

7. Kaliman P, Llagostera E. Myotonic dystrophy protein kinase (DMPK) and its role in the pathogenesis of myotonic dystrophy 1. Cell Signal 2008;20(11):1935-1941.

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8. Pantic B, Trevisan E, Citta A, Rigobello MP, Marin O, Bernardi P, et al. Myotonic dystrophy protein kinase (DMPK) prevents ROS-induced cell death by assembling a hexokinase II-Src complex on the mitochondrial surface. Cell Death Dis 2013;4:e858.

9. Charlet BN, Savkur RS, Singh G, Philips AV, Grice EA, Cooper TA. Loss of the muscle-specific chloride channel in type 1 myotonic dystrophy due to misregulated alternative splicing. Mol Cell 2002;10(1):45-53.

10. Mankodi A, Takahashi MP, Jiang H, Beck CL, Bowers WJ, Moxley RT, et al. Expanded CUG repeats trigger aberrant splicing of ClC-1 chloride channel pre-mRNA and hyperexcitability of skeletal muscle in myotonic dystrophy. Mol Cell 2002;10(1):35-44.

11. Ebralidze A, Wang Y, Petkova V, Ebralidse K, Junghans RP. RNA leaching of transcription factors disrupts transcription in myotonic dystrophy. Science 2004;303(5656):383-387.

12. Mounsey JP, Mistry DJ, Ai CW, Reddy S, Moorman JR. Skeletal muscle sodium channel gating in mice deficient in myotonic dystrophy protein kinase. Hum Mol Genet 2000;9(15):2313-2320.

13. Fu YH, Friedman DL, Richards S, Pearlman JA, Gibbs RA, Pizzuti A, et al. Decreased expression of myotonin-protein kinase messenger RNA and protein in adult form of myotonic dystrophy. Science 1993;260(5105):235-238.

14. Hofmann-Radvanyi H, Junien C. Myotonic dystrophy: over-expression or/and under-expression? A critical review on a controversial point. Neuromuscul Disord 1993;3(5-6):497-501.

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15. Sabourin LA, Tamai K, Narang MA, Korneluk RG. Overexpression of 3'-untranslated region of the myotonic dystrophy kinase cDNA inhibits myoblast differentiation in vitro. J Biol Chem 1997;272(47):29626-29635.

16. Krahe R, Ashizawa T, Abbruzzese C, Roeder E, Carango P, Giacanelli M, et al. Effect of myotonic dystrophy trinucleotide repeat expansion on DMPK transcription and processing. Genomics 1995;28(1):1-14.

17. Gruener R, Stern LZ, Markovitz D, Gerdes C. Electrophysiologic properties of intercostal muscle fibers in human neuromuscular diseases. Muscle Nerve 1979;2(3):165-172.

18. Merickel M, Gray R, Chauvin P, Appel S. Cultured muscle from myotonic muscular dystrophy patients: altered membrane electrical properties. Proceedings of the National Academy of Sciences of the United States of America 1981;78(1):648-652.

19. Kobayashi T, Askanas V, Saito K, Engel WK, Ishikawa K. Abnormalities of aneural and innervated cultured muscle fibers from patients with myotonic atrophy (dystrophy). Arch Neurol 1990;47(8):893-896.

20. Edstrom L, Wroblewski R. Intracellular elemental composition of single muscle fibers in muscular dystrophy and dystrophia myotonica. Acta Neurol Scand 1989;80(5):419-424.

21. Jacobs AE, Benders AA, Oosterhof A, Veerkamp JH, van Mier P, Wevers RA, et al. The calcium homeostasis and the membrane potential of cultured muscle cells from patients with myotonic dystrophy. Biochim Biophys Acta 1990;1096(1):14-19.

22. Benders AA, Wevers RA, Veerkamp JH. Ion transport in human skeletal muscle cells: disturbances in myotonic dystrophy and Brody's disease. Acta Physiol Scand 1996;156(3):355-367.

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23. Ranum LP, Rasmussen PF, Benzow KA, Koob MD, Day JW. Genetic mapping of a second myotonic dystrophy locus. Nat Genet 1998;19(2):196-198.

24. Liquori CL, Ricker K, Moseley ML, Jacobsen JF, Kress W, Naylor SL, et al. Myotonic dystrophy type 2 caused by a CCTG expansion in intron 1 of ZNF9. Science 2001;293(5531):864-867.

25. Meola G. Clinical aspects, molecular pathomechanisms and management of myotonic dystrophies. Acta Myol 2013;32(3):154-165.

26. Chen W, Wang Y, Abe Y, Cheney L, Udd B, Li YP. Haploinsuffciency for Znf9 in Znf9+/- mice is associated with multiorgan abnormalities resembling myotonic dystrophy. J Mol Biol 2007;368(1):8-17.

27. Tan SV, Z'Graggen WJ, Boerio D, Rayan DR, Norwood F, Ruddy D, et al.. Chloride channels in myotonia congenita assessed by velocity recovery cycles. Muscle Nerve 2014;49(6):845-857.

28. Tan SV, Matthews E, Barber M, Burge JA, Rajakulendran S, Fialho D, et al.. Refined exercise testing can aid DNA-based diagnosis in muscle channelopathies. Ann Neurol 2011;69(2):328-340.

29. Boerio D, Z'Graggen WJ, Tan SV, Guetg A, Ackermann K, Bostock H. Muscle velocity recovery cycles: effects of repetitive stimulation on two muscles. Muscle Nerve 2012;46(1):102-111.

30. Tan SV, Z'Graggen W J, Boerio D, Rayan DL, Howard R, Hanna MG, et al. Membrane dysfunction in Andersen-Tawil syndrome assessed by velocity recovery cycles. Muscle Nerve 2012;46(2):193-203.

31. Z'Graggen W J, Bostock H. Velocity recovery cycles of human muscle action potentials and their sensitivity to ischemia. Muscle Nerve 2009;39(5):616-626.

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32. Bostock H, Tan SV, Boerio D, Z'Graggen W J. Validity of multi-fiber muscle velocity recovery cycles recorded at a single site using submaximal stimuli. Clin Neurophysiol 2012;123(11):2296-2305.

33. Bostock H, Baumann C, Humm AM, Z'Graggen W J. Temperature dependency of human muscle velocity recovery cycles. Muscle Nerve 2012;46(2):264-266.

34. Reddy S, Mistry DJ, Wang QC, Geddis LM, Kutchai HC, Moorman JR et al. Effects of age and gene dose on skeletal muscle sodium channel gating in mice deficient in myotonic dystrophy protein kinase. Muscle Nerve 2002;25(6):850-857.

35. Buchanan R, Nielsen OB, Clausen T. Excitation- and beta(2)-agonist-induced activation of the Na(+)-K(+) pump in rat soleus muscle. J Physiology 2002;545(Pt 1):229-240.

36. Shattock MJ. Phospholemman: its role in normal cardiac physiology and potential as a druggable target in disease. Curr Opin Pharmacol 2009;9(2):160-166.

37. Wevers RA, Joosten MG, van de Biezenbos JB, Theewes GM, Veerkamp JH. Excessive plasma K+ increase after ischemic exercise in myotonic muscular dystrophy. Muscle Nerve 1990;13(1):27-32.

38. Andersen G, Orngreen MC, Preisler N, Colding-Jorgensen E, Clausen T, Duno M, et al. Muscle phenotype in patients with myotonic dystrophy type 1. Muscle Nerve 2013;47(3):409-415.

39. Clausen T. Na+-K+ pump regulation and skeletal muscle contractility. Physiol Rev 2003;83(4):1269-1324.

40. Desnuelle C, Lombet A, Serratrice G, Lazdunski M. Sodium channel and sodium pump in normal and pathological muscles from patients with myotonic

For Peer Review Only

muscular dystrophy and lower motor neuron impairment. J Clinical Invest 1982;69(2):358-367.

41. Mounsey JP, John JE, 3rd, Helmke SM, Bush EW, Gilbert J, Roses AD, et al. Phospholemman is a substrate for myotonic dystrophy protein kinase. J Biol Chem 2000;275(30):23362-23367.

42. Benziane B, Widegren U, Pirkmajer S, Henriksson J, Stepto NK, Chibalin AV. Effect of exercise and training on phospholemman phosphorylation in human skeletal muscle. Am J Physiol Endocrinol Metab 2011;301(3):E456-466.

43. Mateos-Aierdi AJ, Goicoechea M, Aiastui A, Fernandez-Torron R, Garcia-Puga M, Matheu A, et al. Muscle wasting in myotonic dystrophies: a model of premature aging. Front Aging Neurosci 2015;7:125.

44. Monckton DG, Caskey CT. Unstable triplet repeat diseases. Circulation 1995;91(2):513-520.

45. Morales F, Couto JM, Higham CF, Hogg G, Cuenca P, Braida C, et al. Somatic instability of the expanded CTG triplet repeat in myotonic dystrophy type 1 is a heritable quantitative trait and modifier of disease severity. Hum Mol Gen 2012;21(16):3558-3567.

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Table 1.

Pt	Diagnosis	Gene	†Size of CTG expansion (DM1)	Gender	Age	TA Power (MRC 1-5)	SET (%)
1	DM1	*DMPK*	medium	W	29	5	28
2	DM1	*DMPK*	medium	W	25	5	77
3	DM1	*DMPK*	NA	W	32	3	26
4	DM1	*DMPK*	medium	M	20	4	12
5	DM1	*DMPK*	small	M	57	5	10
6	DM1	*DMPK*	medium to large	M	49	2	46
7	DM1	*DMPK*	small	W	73	2	10
8	DM1	*DMPK*	NA	M	57	1	21
9	DM1	*DMPK*	medium	M	43	4	20
10	DM1	*DMPK*	NA	W	32	5	20
11	DM1	*DMPK*	large	W	45	3	35
12	DM1	*DMPK*	NA	M	29	5	37
13	DM1	*DMPK*	NA	W	48	4- *	1
14	DM1	*DMPK*	medium	W	20	5	6
15	DM1	*DMPK*	NA	M	30	5	35
16	DM1	*DMPK*	NA	M	59	2	33
17	DM1	*DMPK*	NA	W	54	4- *	
18	DM1	*DMPK*	NA	W	44	4+ *	22
19	DM2	*CNBP*	NA	W	41	5	6.5
20	DM2	*CNBP*	NA	W	67	5	15
21	DM2	*CNBP*	NA	W	57	5	0
22	DM2	*CNBP*	NA	M	38	5	1
23	DM2	*CNBP*	NA	W	62	5	9

*MRC 4- plotted as '3.5', and 4+ as '4.5' for correlations.
† Size of CTG repeats - small: 100-200 repeats, medium: 200-700 repeats, large: >700 repeats. NA= Not available, because the confirmation of the pathological expansion was made using triplet-repeat primed PCR (DM1) or quadruple-repeat primed PCR (DM2), in which no estimation of expansion size is given.
Only patient 14 was on a sodium channel blocker (carbamazepine).

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Table 2. Velocity Recovery Cycle measurements compared between groups.

	NC (n=20)	**DM1 (n=18)**	**DM1 wk (n=8)**	**DM2 (n=5)**
MRRP (ms)	3.96 ± 1.07	3.85 ± 0.84 *P = 0.81*	4.13 ± 1.10 *P = 0.30*	3.78 ± 0.60 *P = 1.0*
ESN (%)	11.1 ± 2.7	9.8 ± 2.5 *P = 0.14*	8.6 ± 2.5 *P = 0.025*	12.4 ± 5.1 *P = 0.82*
ESN@(ms)	8.5 ± 1.9	8.5 ± 1.7 *P = 0.59*	8.9 ± 2.1 *P = 0.50*	8.2 ± 1.0 *P = 0.77*
5ESN (%)	13.0 ± 3.5	10.9 ± 2.8 *P = 0.059*	9.7 ± 3.3 *P = 0.025*	14.7 ± 3.6 *P = 0.45*
SN20 (%)	6.6 ± 1.5	6.5 ± 1.5 *P = 0.98*	5.7 ± 0.9 *P = 0.23*	8.1 ± 2.7 *P = 0.28*
5SN20 (%)	12.5 ± 2.9	10.3 ± 2.7 *P = 0.017*	**9.0 ± 3.2** ***P = 0.0068***	13.6 ± 2.2 *P = 0.53*
LSN (%)	3.7 ± 0.9	3.5 ± 0.9 *P = 0.41*	3.0 ± 0.8 *P = 0.089*	4.2 ± 1.2 *P = 0.53*
RSN (%)	0.17 ± 0.20	0.12 ± 0.28 *P = 0.97*	0.09 ± 0.37 *P = 0.64*	0.13 ± 0.40 *P = 0.87*
5XLSN (%)	7.3 ± 1.7	7.0 ± 1.8 *P = 0.55*	7.0 ± 2.0 *P = 0.75*	8.3 ± 1.1 *P = 0.30*
5XRSN (%)	0.98 ± 0.36	**2.37 ± 0.99** ***P = 0.00000021***	**2.27 ± 1.08** ***P = 0.00039***	**2.25 ± 0.85** ***P = 0.0023***

The first column shows values obtained from the tibialis anterior muscle in 20 normal control subjects (NC). Columns 2 and 4 show values obtained from the DM1 and DM2 patients. Column 3 shows values from the subset of the 8 DM1 patients in whom the tibialis anterior was weak (MRC grades <4). *P* values are for comparisons with the normal control group. Significant *P* values of <0.01 are highlighted.

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Table 3. Frequency Ramp measurements compared between groups.

	NC (n=20)	DM1 (n=18)	DM1 wk (n=8)	DM2 (n=5)
Lat(15Hz)$_{Last}$%	84.5 ± 3.8	84.2 ± 4.4 *P = 0.56*	83,0 ± 4.4 *P = 0.31*	84.6 ± 9.0 *P = 0.37*
Lat(15Hz)$_{First}$%	93.5 ± 3.1	93.4 ± 3.9 *P = 0.87*	93.3 ± 3.2 *P = 0.94*	93.5 ± 1.6 *P* = 0.68
Lat(30Hz) $_{First}$ %	94.6 ± 4.0	94.6 ± 5.4 *P = 0.87*	94.2 ± 6.3 *P = 0.55*	94.9 ± 2.3 *P = 1.0*
Lat(30Hz+30s)%	101.7 ± 1.7	100.6 ± 2.9 *P = 0.26*	**98.4 ± 2.4** ***P = 0.000061***	101.4 ± 2.3 *P = 0.78*

Effects of frequency ramp on latencies of muscle action potentials. Values given are mean ± SD, *P* values are for the Mann Whitney U test. Significant *P* values of <0.01 are highlighted.

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Figure legends

Figure 1. Muscle velocity recovery cycles following 1 and 5 conditioning impulses. Mean changes in latency with time after single conditioning impulse (**A**) and 5 conditioning impules (**B**) for 3 groups superimposed: grey line = 20 NC, filled circles = 18 DM1, filled triangles = 5 DM2. **C**: Latency changes for 5 different groups 20 ms after 5 conditioning impulse, with the DM1 group separated into stronger (10s) and weaker (8w) subgroups. Boxes indicate interquartile ranges, and lines indicate median values. Comparisons with NC group by Mann Whitney U test indicated as NS = $P>0.05$, * = $P<0.05$, ** $P<0.01$, *** = $P<0.001$, **** = $P < 0.0001$, ***** = $P<0.00001$. **D**: Similar plots for residual supernormality, 900-1000 ms after 5 conditioning stimuli. Arrows in B indicate times of measurements in C and D.

Figure 2. Effect of sodium channel blockers on MVRCs in myotonia congenita. Scatter plots of 4 groups, comparing early and late supernormality changes as in Fig. 1C and D, between NC, DM1, and MC patients not on medication (MC^a), and MC patients being treated with sodium channel blockers (MC^b).

Figure 3. Effects of frequency ramp on velocities of muscle action potentials. Left hand column shows latency changes from baseline stimulation (0.5 Hz) to first stimuli in 1 sec trains, that increased from 1 to 30 Hz, and recovery when stimulation reverted to 0.5 Hz. Grey lines: mean of NC group. Black circles: mean values for stronger DM1 patients (**A**), weaker DM1 patients (**B**), and DM2 patients (**C**). **D**: Latency changes 30 sec after end of frequency ramp for 5 groups plotted as in Fig. 1C; **** = $P<0.0001$. **E**: Correlation between latency changes 30 sec after frequency ramp and muscle strength according to MRC scale, for 18 DM1 patients; *** = $P<0.001$.

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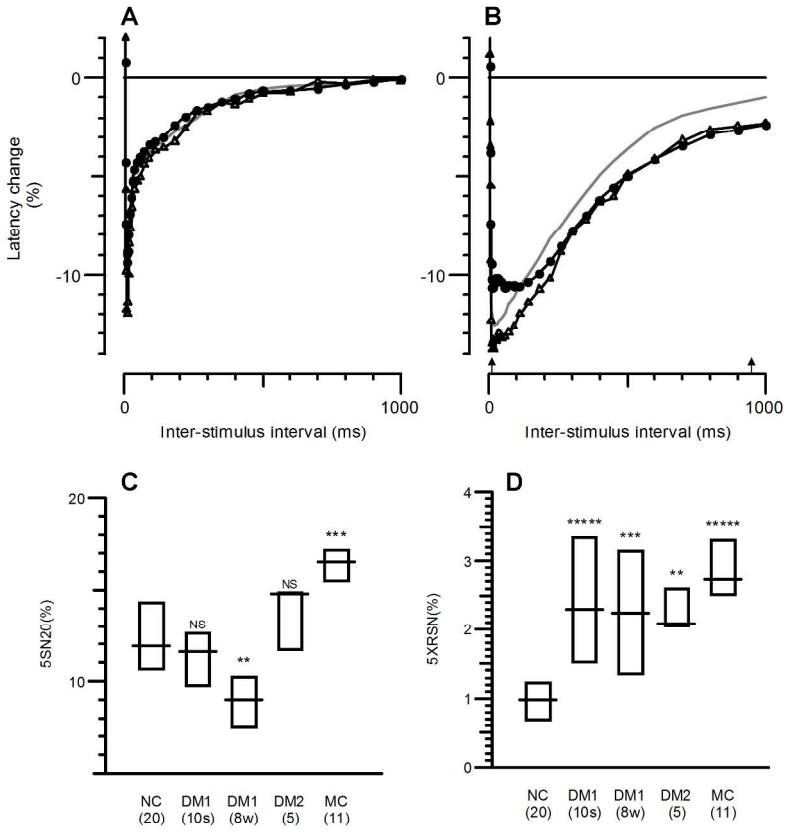

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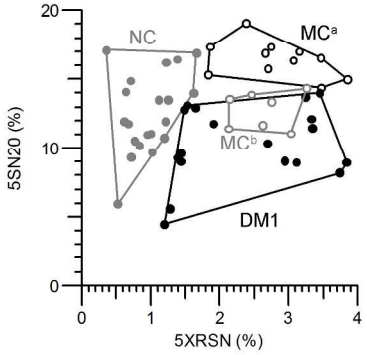

Figure 2. Effect of sodium channel blockers on MVRCs in myotonia congenita. Scatter plots of 4 groups, comparing early and late supernormality changes as in Fig. 1C and 1D, between NC, DM1 and MC patients not on medication (MCa), and MC patients being treated with sodium channel blockers (MCb).
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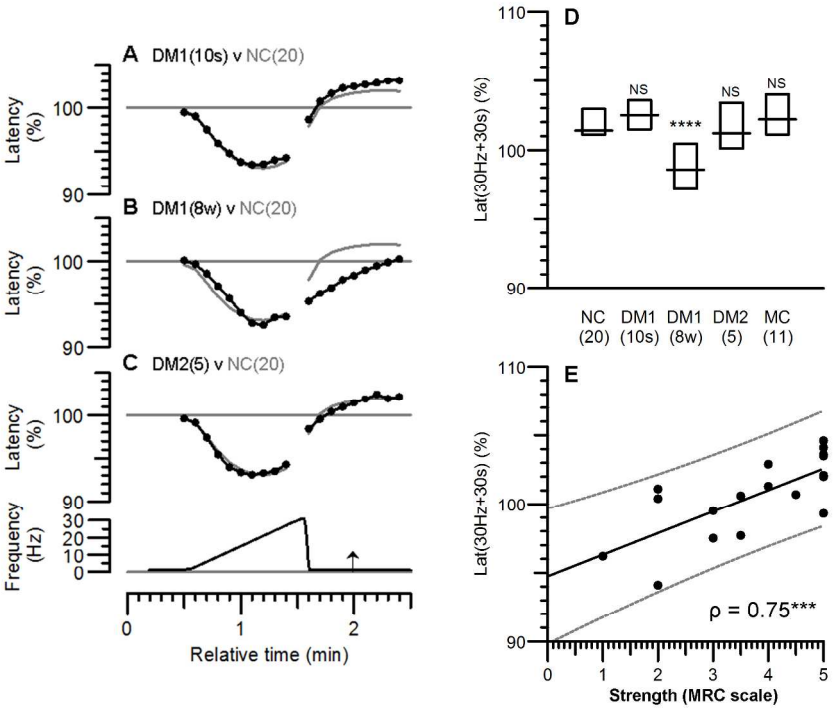

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