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Short communication

# POTASSIUM CURRENTS IN HUMAN MYOGENIC CELLS FROM HEALTHY AND CONGENITAL MYOTONIC DYSTROPHY FOETUSES

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**Abstract:** The whole-cell patch clamp technique was used to record potassium currents in *in vitro* differentiating myoblasts isolated from healthy and myotonic dystrophy type 1 (DM1) foetuses carrying 2000 CTG repeats. The fusion of the DM1 myoblasts was reduced in comparison to that of the control cells. The dystrophic muscle cells expressed less voltage-activated $K^+$ (delayed rectifier and non-inactivating delayed rectifier) and inward rectifier channels than the age-matched control cells. However, the resting membrane potential was not significantly different between the control and the DM1 cells. After four days in a differentiation medium, the dystrophic cells expressed the fast-inactivating transient outward $K^+$ channels, which were not observed in healthy cells. We suggest that the low level of potassium currents measured in differentiated DM1 cells could be related to their impaired fusion.

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Abbreviations used: DM1 – myotonic dystrophy type 1; DMED – differentiation medium; DMPK – dystrophia myotonica protein kinase; $I_{K(DR)}$ – delayed rectifier current; $I_{K(IR)}$ – inward rectifier current; $I_{K(TO)}$ – transient outward $K^+$ current; TEACl – tetraethylammonium chloride

**Key words**: Potassium channels, Myoblast fusion, Congenital myotonic dystrophy, Patch-clamp

## INTRODUCTION

Myotonic dystrophy type 1 (DM1), the most common type of adult-onset muscular dystrophy, was first described in 1909 as a distinct pathology by Steinert, Batten and Gibb. DM1 patients suffer from myotonia (skeletal muscle membrane hyperexcitability), muscle weakness and progressive muscle wasting, and a wide range of highly variable symptoms in multiple tissue systems. The DM1 mutation has been identified as an expanded CTG trinucleotide repeat in the 3'-untranslated region of the dystrophia myotonica protein kinase (*DMPK*) gene [1-3] on chromosome 19. The severity of disease onset in DM1 patients is correlated with the number of CTG repeats, which can reach several thousand [1]. The transcription of the mutant allele does not seem to be affected, but *DMPK* transcripts containing the CUG expansion, in contrast to the normal *DMPK* transcripts, accumulate as discrete foci in the nucleus [4-6]. These nuclear aggregates are possibly involved in altering the functions of several RNA-binding proteins such as MBNL (muscleblind-like) and CELF (CUG-BP1 and ETR-3-like factors), leading to abnormal splicing of several target pre-mRNAs, as shown for the insulin receptor and the muscle-specific chloride channel (ClC-1) [7-9]. This mechanism of RNA gain-of-function clearly contributes to DM1 pathogenesis.

There are now several pieces of experimental evidence suggesting that myotonia could be due to a decrease in the expression of muscle-specific ClC-1 chloride channels [8, 10]. Moreover, an increased cytosolic $Ca^{2+}$ concentration [11], altered expression of small conductance $Ca^{2+}$-activated $K^+$ channels [12] and a gating abnormality of $Na^+$ channels [13, 14] could also contribute to the modifications in the excitability in DM1 muscle cells. However, the muscle wasting observed during the progression of the disease and the severe impairment of muscle maturation described in the severe congenital form of DM1 [15, 16] cannot be explained by these electrophysiological observations. Muscle biopsies from congenital foetuses display a decreased number of satellite cells (muscle stem cells) [17], and alterations in the behavior of human satellite cells isolated from congenital DM1 patients with a large CTG expansion have been shown *in vitro* [18, 19]. The key role of the CUG expansion in the impaired myogenic differentiation was confirmed in the C2C12 culture model system over-expressing the 3'UTR of *DMPK* with 200 CTG repeats [20].

In healthy muscles, satellite cells (present beneath the basal lamina surrounding each myofiber) are quiescent until muscle damage occurs. Once activated, these cells start to proliferate as myoblasts and then differentiate and fuse into newly formed myotubes or damaged fibers. Electrophysiological studies performed on healthy human myoblasts indicated that the appearance of voltage-gated $K^+$ channels and the setting of the resting membrane potential (RMP) in the

vicinity of -60 mV are processes associated with cell fusion [21-24]. Therefore, it has been proposed that cell hyperpolarization, associated with the expression of two $K^+$ currents, $I_{K(NI)}$, a non-inactivating delayed rectifier, and $I_{K(IR)}$, an inward rectifier, is involved in the mechanism of $Ca^{2+}$ influx, an event observed prior to the cell fusion of human myoblasts [23]. Cells expressing the $K_{NI}$ and $K_{IR}$ channels are then able to achieve a more negative RMP than proliferating cells, allowing T-type $Ca^{2+}$ channels to conduct a $Ca^{2+}$ window current [25]. Since these voltage-gated $K^+$ currents are indeed crucial for cell fusion, we decided to determine their activity in DM1 muscle cells.

## MATERIALS AND METHODS

### Human satellite cells

Human satellite cells were isolated from quadriceps muscle biopsies of 28-week old foetuses and established in cell culture as described previously [14, 18]. The biopsies were obtained during autopsies with the authorization of the local ethical committee and in accordance with French legislation and ethical rules. The control foetus was devoid of any signs of neuromuscular disease, while the DM1 foetus displayed clinical evidence of the congenital form of muscular dystrophy (~2000 CTG repeats, bilateral varus deformation of the feet, muscle hypotrophy and arthrogryposis: multiple joint contractures at birth). Satellite cells were isolated and grown as myoblasts as described previously [26].

### Cell culture

Human myoblasts were cultured in a growth medium composed of Dulbecco's modified Eagle's nutrient medium (DMEM) supplemented with 20% foetal calf serum, 1 mM L-glutamine, 100 units $ml^{-1}$ penicillin and 100 μg $ml^{-1}$ streptomycin (all from ICN Biomedicals). The cultures were incubated at 37ºC in a humid air atmosphere in 5% $CO_2$-enriched air. To induce myoblast differentiation and fusion, the cells were plated at a density of 8000 cells/$cm^2$, and 2-3 days after the plating, the growth medium was replaced with a differentiation medium (DMED) composed of DMEM supplemented with 4 mM L-glutamine, 100 units $ml^{-1}$ penicillin, 100 μg $ml^{-1}$ streptomycin and 4% horse serum (ICN).

### Electrophysiological recordings

Cells grown in differentiation medium for 2-6 days were used. Large myotubes (with a membrane input capacitance > 100 pF) and round myoblasts were excluded from the experiments. The bath solution contained: 144 mM NaCl, 2.8 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, and 2 mM EGTA, with the pH adjusted to 7.4 with NaOH. In some experiments, 97 mM NaCl was replaced with 90 mM TEACl and 5 mM $BaCl_2$ to block the $K^+$ currents; in others, 1 μM TTX was added to the medium to block fast $Na^+$ currents. The pipette solution contained: 141 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, and 11 mM EGTA, pH 7.4. All the chemicals were from Sigma-Aldrich. The pipette

resistances were 2-5 MΩ. The resting membrane potential (RMP), i.e. the potential of the cell interior with respect to an external zero potential, was measured in the current-clamp mode immediately after the formation of the seal. The electrophysiological recordings were performed at room temperature (~23ºC). Ionic currents were recorded in the whole-cell configuration of the patch-clamp technique, using an Axopatch 200B amplifier; the sampling rate was 10 kHz, and the signals were low-pass filtered at 2 kHz. The whole-cell capacitance was compensated for. The series resistance was typically less than 10 MΩ. The currents were analyzed using Axon Instruments Clampfit 7 software (Molecular Devices Corporation, CA, USA). The time constants of inactivation were estimated from single or double exponential fits of the membrane currents from the peak of the current to the end of the depolarizing pulse. In most of the experiments, the holding potential ($V_{hold}$) was -60 mV, from which depolarizing or hyperpolarizing voltage steps were applied (potential increment ± 10 mV, pulse duration 600 ms, time interval between pulses 6-9 s). The current density (pA $pF^{-1}$) was calculated as the current peak at +40 mV or at -140 mV divided by the cell input capacitance.

**DAPI nuclei count**

Cells were fixed in freshly prepared 3.7% paraformaldehyde in phosphate buffered saline solution (PBS) for 15 min at 37ºC, and permeabilised by a 5-min incubation in 100% methanol at 37ºC. The nuclei were labeled using 4,6-diamino-2-phenylindole (DAPI). Staining was carried out by incubating cells for 10 min at 37ºC in PBS containing 10 μM DAPI. The cells were then rinsed in PBS, and the fluorescence images were compared to the corresponding bright-field images in some preparations in order to identify the cell types. The fusion index is the percentage of myotubes (cells with three or more nuclei).

**Statistical analysis**

The results are expressed as means ± SEM. A Student's *t*-test was applied to compare the means of two groups. To compare the non-Gaussian variables (time constants of current inactivation), we applied a Mann-Whitney test. To compare proportions, we used the contingency table method, and the two-tailed *P* value was calculated by Fisher's exact test. Differences were considered significant when $P < 0.05$.

## RESULTS

**Fusion of muscle cells**

During the proliferation stage, no differences in morphology were observed between the control and DM1 cells. After 6 days in the DMED differentiation medium, the percentage of multinucleated cells was significantly lower in the population of DM1 cells than in the population of healthy cells (Fig. 1, $P < 0.01$). The DM1 cells had no more than four nuclei, while in the control cells, up to 15 nuclei were observed (data not shown).

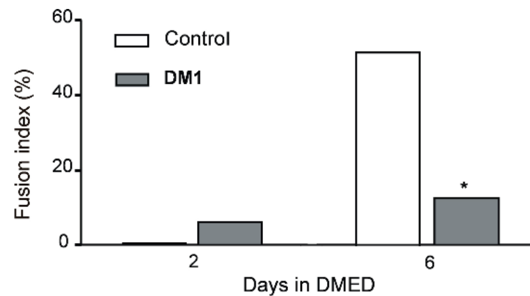


Fig. 1. The mean fusion index (per cent of cells with at least 3 nuclei) after transfer to a differentiation medium (DMED). *Significant difference relative to the control ($P < 0.001$; Fisher's exact test); between 49 and 95 cells were counted in a given experiment.

**Cell membrane capacitance**

Cell capacitance was measured immediately after the formation of the whole-cell configuration. In both groups of cells, the mean capacitance on day 2 (37.0 ± 3.5 pF, $n = 11$ for the healthy cells, and 44.3 ± 4.6 pF, $n = 27$ for the dystrophic cells) differed significantly ($P < 0.005$) from the mean capacitance calculated on day 6 (74.8 ± 5.9 pF, $n = 15$ for the healthy cells, and 65.9 ± 5.4 pF, $n = 20$ for the dystrophic cells). However, the differences between the mean capacitance of the healthy and DM1 cells compared on the same days were insignificant. This lack of difference may result from a technical limitation of the whole-cell technique, as the largest cells, which were numerous in the healthy cell population, were excluded from measurements.

**Resting membrane potential**

The resting membrane potential (RMP) was measured in the healthy control and DM1 muscle cells grown in the differentiation medium for 2, 4 or 6 days. In both groups, strongly and weakly hyperpolarized cells coexisted throughout the experiment. The mean RMPs were -35.0 ± 3.5 mV (healthy cells) and -38.5 ± 3.0 mV (DM1 cells) on day 2 and -47.9 ± 15.0 mV (healthy cells) and -41.4 ± 10.0 mV (DM1 cells) on day 6. The mean RMP value in the group of healthy cells averaged over days 2 to 6 was -38.4 ± 2.2 mV ($n = 51$), and in the group of DM1 cells over the same days was -35.2 ± 2.0 mV ($n = 47$). No statistically significant differences were therefore observed between the mean RMP values obtained for the healthy and DM1 cells under our experimental conditions.

**Outward-rectifying $K^+$ currents**

Upon membrane depolarization, both the healthy control cells (47 out of 51; 92%) and DM1 cells (65 out of 80; 81%) grown in the differentiation medium for 2, 3, 4 or 6 days showed outwardly-rectifying $K^+$ currents. Three outward rectifier-type $K^+$ currents were observed: a slowly inactivating delayed rectifier

$I_{K(DR)}$ current, a non-inactivating outward rectifier $I_{K(NI)}$ current, and a fast inactivating transient outward rectifier $I_{K(TO)}$ current (Fig. 2).

The mean time constant of inactivation of $I_{K(DR)}$ was 359 ± 119 ms for the control ($n$ = 26) and 358 ± 144 ms ($n$ = 31) for the DM1 cells. To determine the non-inactivating $I_{K(NI)}$ current, a 15-s depolarizing pulse to +40 mV was used to inactivate the $I_{K(DR)}$ channels. In the healthy cells, the current density of $I_{K(DR)}$ and $I_{K(NI)}$ increased with the duration of differentiation up to day 4; such a tendency was not observed in the DM1 cells. Moreover, on all days, the DM1 cells showed a significantly lower mean density of the $I_{K(DR)}$ current compared with the healthy cells (Fig. 3). After four days in DMED, the $I_{K(DR)}$ current

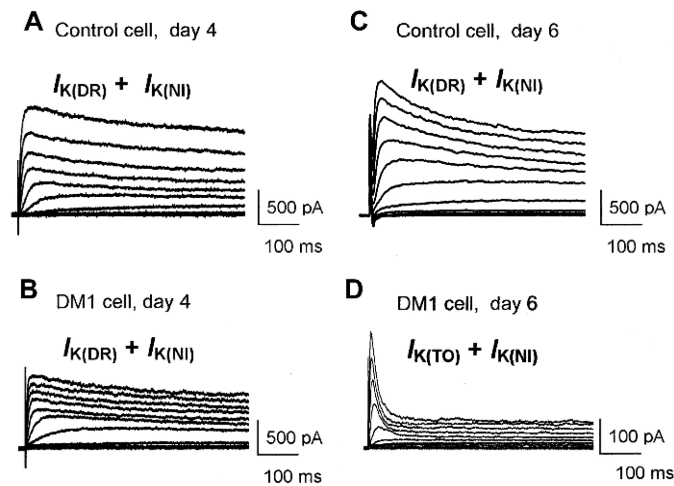


Fig. 2. Examples of the outward $K^+$ currents recorded from the control and DM1 cells. A, B – day 4; $V_{hold}$ = -60 mV, potential steps 10 mV to +40 mV; no leak subtraction. C, D – day 6; $V_{hold}$ = -90 mV, potential steps 15 mV to +45 mV; no leak subtraction.

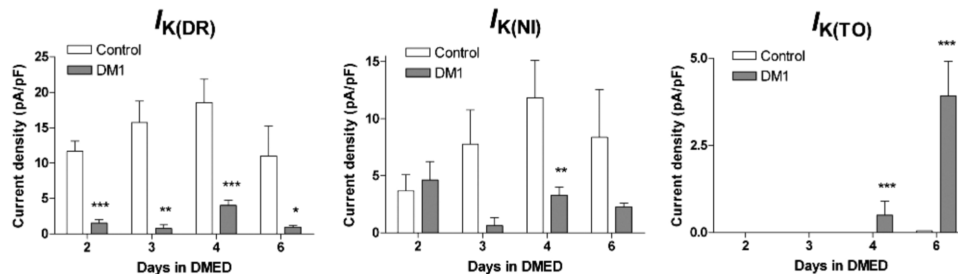


Fig. 3. The densities of the evoked outward $K^+$ currents ($I_{K(DR)}$, $I_{K(NI)}$ and $I_{K(TO)}$) during differentiation in DMED medium measured after changing the potential from -60 mV to +40 mV. The data is the means ± SEM. 6 to 27 cells were measured. **$P < 0.01$, and ***$P < 0.001$ were considered significant differences between the control and DM1 cells (Student's $t$-test).

started to be replaced by a transient $I_{K(TO)}$ current in the DM1, but not in the control cells (Fig. 2).

The mean time constant of $I_{K(TO)}$ inactivation (18.1 ± 1.9 ms; *n* = 12) differed significantly from the mean time constant of $I_{K(DR)}$ inactivation (Mann-Whitney test, $P < 0.0004$). In four DM1 cells expressing the $I_{K(TO)}$ current, we applied two different holding potentials (-60 mV and -90 mV) before inducing membrane currents (Fig. 4). At both these holding potentials, the $I_{K(TO)}$ currents were present, and had similar peak amplitudes. All the types of outward-rectifying current were abolished in the presence of the $K^+$ channel blockers 90 mM TEACl + 5 mM $BaCl_2$ (*n* = 4 experiments for each cell type; data not shown). TTX (1 μM) had no effect on the observed $K^+$ currents.

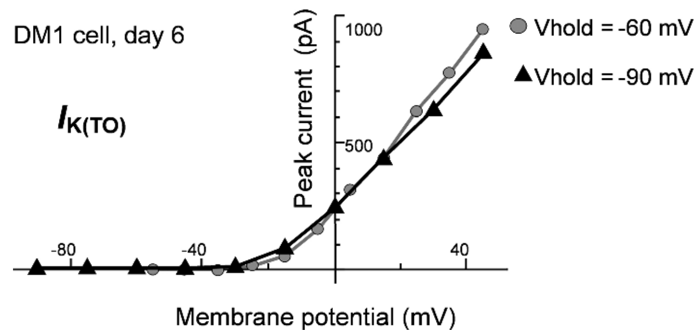


Fig. 4. The peak *I-V* relation measured in a DM1 cell on day 6, applying a $V_{hold}$ of -60 mV or -90 mV. Note the similar peak amplitudes of ($I_{K(TO)}$) under both conditions. The slightly lower amplitude of the current measured at positive potentials from a $V_{hold}$ of -90 mV may have resulted from a small, undetected $Na^+$ current. All the currents were recorded in the presence of 1 μM TTX. The leakage current was subtracted from the experimental data.

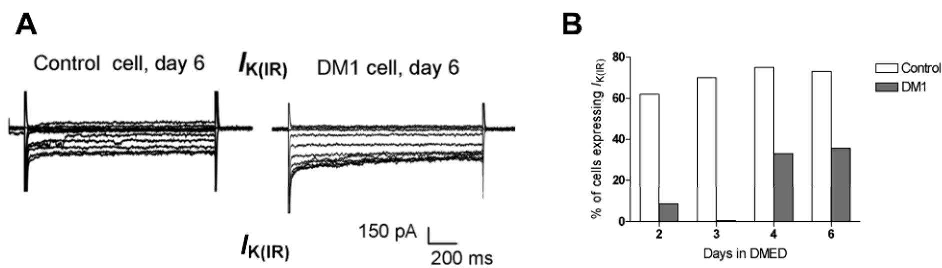


Fig. 5. The inward-rectifying $K^+$ current ($I_{K(IR)}$). A - $I_{K(IR)}$ recorded from a control and a DM1 cell on day 6 in DMED; $V_{hold}$= -60 mV, potential steps -10 mV to -140 mV; no leak subtraction. B – Percentage of cells expressing $I_{K(IR)}$; between 7 and 24 cells were counted in a given experiment.

## Inward rectifier $K^+$ current ($I_{K(IR)}$)

In 69% of the healthy cells (*n* = 41) and 19% of the DM1 cells (*n* = 57) on days 2-6 in DMED, applying hyperpolarizing voltage steps more negative than -90 mV (up to -140 mV) elicited an inward-rectifying $K^+$ current ($I_{K(IR)}$; Fig. 5).

The current was abolished by 0.5 mM $BaCl_2$ (data not shown). No statistically significant differences were observed between the mean densities of $I_{K(IR)}$ in the DM1 and healthy cells (the means refer only to the cells expressing the $I_{K(IR)}$ current).

## DISCUSSION

We investigated voltage-gated $K^+$ currents expressed during the *in vitro* differentiation of human satellite cells derived from the muscles of a healthy control subject and a subject affected by congenital DM1 with 2000 CTG repeats in the *DMPK* gene. We found that the DM1 myoblasts had a lower ability to form multinucleated myotubes compared to the healthy myoblasts, thus confirming the defect in myogenic fusion already observed in DM1 cells [6]. We also observed that in both types of cell, the RMP changed to more negative values during differentiation. However, since throughout the experiment, strongly hyperpolarized cells coexisted with weakly hyperpolarized ones, the calculated mean changes in RMP between different days of differentiation or between the control and DM1 cells were not statistically significant. Moreover, since the whole-cell technique changes the physiological ion composition inside the cell, a less invasive technique would be necessary to measure the RMP of intact cells.

Interestingly, we found that DM1 muscle cells expressed less voltage-gated $K^+$ currents ($I_{K(DR)}$, $I_{K(NI)}$) and less of the $I_{K(IR)}$ current than healthy cells, and that the DM1 cells also expressed a fast-inactivating transient outward $K^+$ current ($I_{K(TO)}$) that was almost absent in the healthy cells. $I_{K(TO)}$ was expressed starting from day 4 in the differentiation medium, and was clearly visible on day 6. A similar current was observed in the normal smooth muscle cells [27] and cardiac myocytes [28] but not in the skeletal muscle cells [29]. The only fast-inactivating $K^+$ current observed in the skeletal myoblasts is the $Na^+$-activated $K^+$ current [30]. $I_{K(TO)}$ was observed even after the $Na^+$ currents were suppressed by applying TTX. The $K_{TO}$ channel differs from the more common A-type $K^+$ channel, which inactivates completely at potentials around -55 mV [31]. In DM1 cells, a similar holding potential, -60 mV, had no effect on the $I_{K(TO)}$ current amplitude, so it is unlikely that such channels are expressed in the DM1 cells. Molecular biology techniques will be needed to identify the potassium channel subtypes expressed during the differentiation of dystrophic cells.

In human muscle cells, $K^+$ channels are important for myotube formation. It has been demonstrated that inhibiting the Kir2.1 inward rectifier channel expression prevents the formation of multinucleated myotubes [24]. It has also been shown that the $K^+$ channel blocker TEA impaired myotube formation, suggesting that TEA-sensitive channels (either $K_{DR}$ and $K_{NI}$ together, or only one of them) are necessary for myoblast fusion [32]. We suggest that the low $K^+$ currents measured during the differentiation of DM1 cells could be implicated as a cause of the impaired fusion of the DM1 cells. Since the expression of the $K_{DR}$ and $K_{IR}$

channels was already significantly lower in the DM1 cells on day 2 of differentiation, it would be interesting to determine the mechanism linking the primary events of the DM1 mutation (i.e. nuclear foci or DMPK haploinsufficiency) with the impaired $K^+$ channel expression in DM1 cells. Although our data should be considered as preliminary, since cells from only two donors were used, we suggest that the DM1 pathology affects the expression of voltage-gated $K^+$ channels in muscle cells.

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