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Myotonic Dystrophy Protein Kinase Is Involved in the Modulation of the Ca\textsuperscript{2+} Homeostasis in Skeletal Muscle Cells

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Abstract

Myotonic dystrophy (DM), the most prevalent muscular disorder in adults, is caused by (CTG)\textsubscript{n}-repeat expansion in a gene encoding a protein kinase (DM protein kinase; DMPK) and involves changes in cytoarchitecture and ion homeostasis. To obtain clues to the normal biological role of DMPK in cellular ion homeostasis, we have compared the resting \( [\text{Ca}^{2+}] \text{J} \) of wild-type and DMPK\textemdash\textemdash knockout mice.

In vitro-differentiated DMPK\textemdash\textemdash knockout myotubes exhibit a higher resting \( [\text{Ca}^{2+}] \text{J} \) than do wild-type myotubes because of an altered open probability of voltage-dependent L-type \( \text{Ca}^{2+} \) channels. The mutant myotubes exhibit smaller and slower \( \text{Ca}^{2+} \) transients, and the content of ATP-driven ion pumps in cultured skeletal muscle cells of wild-type and DMPK\textemdash\textemdash knockout mice.

In conclusion, our data suggest that DMPK is involved in modulating the initial events of excitation-contraction coupling in skeletal muscle. (J. Clin. Invest. 1997. 100:1440-1447.) Key words: excitation-contraction coupling \cdot ion channels \cdot ion pumps \cdot knockout mouse \cdot cultured myotubes

Introduction

Myotonic dystrophy (DM) follows an autosomal dominant inheritance, and is characterized by a wide variety of symptoms, including increased excitability and delayed relaxation of muscle, muscle weakness and wasting, ocular cataract, sensorineural deafness, cardiac conduction defects, hypsarrhythmia, testicular atrophy causing male sterility, and endocrine dysfunction (1). Clinical manifestation of this rather frequent disorder is caused by expansion of an unstable CTG-repeat in the 3'-untranslated region of a gene encoding the myotonic dystrophy protein kinase (DMPK), with the age of onset and the severity of the disease being correlated to the extent of expansion (2-4).

Contradictory results have been obtained with regard to the effects of abnormally long CTG repeats on DMPK mRNA and protein levels in patient tissues (5-8). In addition, the study of transgenic mouse models with altered DMPK levels (9, 10) has not provided the answer on the question whether over- or underexpression of this protein is involved in disease etiology. Homozygous DMPK\textemdash\textemdash knockout mice exhibit only minor changes in neck muscle fibres at older age, whereas animals carrying multiple copies of the DMPK transgene show hypertrophic cardiomyopathy and enhanced neonatal mortality as the only features.

Typically, muscle fibres and/or cultured skeletal muscle cells of DM patients exhibit a decreased resting membrane potential (11-13) and increased basal cytosolic Na\textsuperscript{+} and \( \text{Ca}^{2+} \) concentrations (14-16). These features may be attributed to anomalies in the functioning of voltage-operated Na\textsuperscript{+} channels (17, 18) and \( \text{Ca}^{2+} \) channels (15, 16) and/or to a reduced content of \( \text{Na}^{+}/\text{K}^{+}-\text{ATPase} \) and sarcoplasmic reticulum \( \text{Ca}^{2+} \)-ATPase are affected by DMPK absence.

In conclusion, our data suggest that DMPK is involved in modulating the initial events of excitation-contraction coupling in skeletal muscle. (J. Clin. Invest. 1997. 100:1440-1447.) Key words: excitation-contraction coupling \cdot ion channels \cdot ion pumps \cdot knockout mouse \cdot cultured myotubes

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1. Abbreviations used in this paper: \( \text{ACh(R)} \), acetylcholine (receptor); \( \text{DHPR} \), dihydropyridine receptor; DM, myotonic dystrophy; DMPK, myotonic dystrophy protein kinase; E-C, excitation-contraction; \( \text{RyR} \), ryanodine receptor; SR, sarcoplasmic reticulum; \( \text{TTX(R)} \), tetrodotoxin (receptor); \( \tau_d \), half-decay time; \( \tau_i \), half-increase time; \( 3\text{-O-MFPase} \), 3-O-methylfluorescein phosphatase.
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Table 1. Ca2+ Transients Induced by ACh or KCl in Cultured Skeletal Muscle Cells From Wild-type and DMPK[-/-] Mice

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Parameter</th>
<th>Wild-type</th>
<th>DMPK[-/-]</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>[Ca2+]max</td>
<td>122±17</td>
<td>185±24*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(170)</td>
<td>(63)</td>
</tr>
<tr>
<td>20 µM ACh</td>
<td>[Ca2+]max</td>
<td>775±131</td>
<td>511±124*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(84)</td>
<td>(38)</td>
</tr>
<tr>
<td>125 mM KCl</td>
<td>[Ca2+]max</td>
<td>881±164</td>
<td>552±130*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(86)</td>
<td>(25)</td>
</tr>
</tbody>
</table>

Values are means±SD of the number of muscle cells examined (listed between parentheses) from at least four individual cultures. The valves of [Ca2+] are expressed in nM, and the half-increase (τi) or half-decay time (τd) in s. Parameters of DMPK[-/-] and wild-type cells differ with *P < 0.01.
Figure 1. Depolarization-induced Ca$^{2+}$ transients in cultured skeletal muscle cells derived from wild-type (solid line) and DMPK[-/-] mice (dashed line). Depolarization was generated by 20 µM ACh (A) or 125 mM KCl (B) as marked by the bars. Traces show the average [Ca$^{2+}$], after superimposing all appropriate experiments as listed in Table I.

age-operated Na$^+$ channels in cultured skeletal muscle cells (28, 32). The K$^+$-triggered Ca$^{2+}$ transients are not affected by tetrodotoxin (Fig. 4 C), as expected. It is important to note that neither of the three inhibitors have any effect on the resting [Ca$^{2+}$]$_i$ (Figs. 2 A, 3 A, and 4 A).

Depolarization of the cultured muscle cells in the absence of extracellular Ca$^{2+}$ could not be performed, since the omission of external Ca$^{2+}$ before depolarization provokes a Ca$^{2+}$ transient (data not shown). As a result, the SR becomes at least partially depleted for Ca$^{2+}$, which in turn can affect the kinetics of the SR Ca$^{2+}$ release channel (33). In addition, the DHPR may become inactivatable due to a loss in occupancy of its priming site (23, 25).

Ca$^{2+}$ homeostasis of DMPK[-/-] skeletal muscle cells. Surprisingly, the resting [Ca$^{2+}$]$_i$ (185 nM) of DMPK[-/-] skeletal muscle cells in culture is significantly higher than in wild-type cells (Table I, Fig. 1). Ryanodine does not affect this elevated level (Fig. 2 A), but nifedipine and tetrodotoxin normalize the increased resting [Ca$^{2+}$], completely or partially, respectively (Figs. 3 A and 4 A). This means that DHPRs and TTXRs are constitutively open in resting mutant skeletal muscle cells, unlike in wild-type cells. Strikingly, the amplitudes of the Ca$^{2+}$ responses provoked by depolarization with ACh or KCl are reduced by about 40%, whereas $\tau_1$ and $\tau_2$ are increased 2- and 1.6-fold, respectively (Fig. 1). We observed that ryanodine reduces the depolarization-induced Ca$^{2+}$ responses in DMPK[-/-] cells by only 90% (Fig. 2, B and C), whereas complete blocking is achieved with nifedipine (Fig. 3, B and C). This observation indicates that the Ca$^{2+}$ transients in these cells partially result from an influx of extracellular Ca$^{2+}$ through the DHPRs. Tetrodotoxin incompletely inhibits ACh-evoked Ca$^{2+}$ transients like in wild-type cells (Fig. 4 B). After inhibition of TTXR by tetrodotoxin, when the basal [Ca$^{2+}$]$_i$ in DMPK[-/-] cells has become lowered, the KCl-generated Ca$^{2+}$ responses are both higher and faster than in the absence of tetrodotoxin. The average amplitude of the Ca$^{2+}$ transients raises ~140% and $\tau_1$ and $\tau_2$ are reduced 1.5- and 1.6-fold, respectively, and become similar to the values in wild-type cells.

Figure 2. Effects of ryanodine on resting [Ca$^{2+}$] (A) and depolarization-provoked Ca$^{2+}$ responses (B and C) in cultured wild-type and DMPK[-/-] muscle cells. Muscle cells were incubated with 10 µM ryanodine for 30 min before depolarization (B and C). Traces show the mean [Ca$^{2+}$], after superimposing all relevant experiments as given in Table II. Ryanodine does not normalize the higher resting [Ca$^{2+}$] of DMPK[-/-] cells (A), and only partially inhibits depolarization-induced Ca$^{2+}$ transient (B and C).

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Figure 3. Effects of nifedipine on resting \([\text{Ca}^{2+}]_j\) (A) and depolarization-generated \(\text{Ca}^{2+}\) transients (B and C) in cultured wild-type and DMPK\([-/-]\) muscle cells. Muscle cells were incubated with 5 \(\mu\text{M}\) nifedipine for 30 min before depolarization (B and C). For other details, see legend to Fig. 2. Nifedipine completely normalizes the higher resting \([\text{Ca}^{2+}]_j\) of DMPK\([-/-]\) cells (A).

(compare Figs. 1 B and 4 C). Since these latter observations suggest a \([\text{Ca}^{2+}]_i\)-regulated fine-tuning of depolarization-induced \(\text{Ca}^{2+}\) responses, we investigated whether there was any relation between \([\text{Ca}^{2+}]_i\) and any of the parameters that describe the shape and the magnitude of the \(\text{Ca}^{2+}\) transients in both cell types. The calculations clearly demonstrate that the resting \([\text{Ca}^{2+}]_i\) inversely correlates with \([\text{Ca}^{2+}]_{i\text{max}}\) (Fig. 5 A), and exhibits a high correlation with \(\tau_0\) (Fig. 5 B). In addition, an inverse correlation exists between \([\text{Ca}^{2+}]_{i\text{max}}\) and \(\tau_0\) (Fig. 5 C).

The statistical evaluations of the depolarization-induced \(\text{Ca}^{2+}\) transients of both wild-type and mutant skeletal muscle cells in the absence or presence of ion channel-specific inhibitors are listed in Tables I and II, respectively, and sustain the described phenomena.

\(\text{SR Ca}^{2+}\)-ATPase and Na\(^+\)/K\(^+\)-ATPase. The here observed \(\text{Ca}^{2+}\) homeostasis of cultured DMPK\([-/-]\) mouse muscle cells has the same characteristics as in cultured skeletal muscle cells derived from DM patients (15, 16). Since muscle and cultured muscle cells of patients have been shown to exhibit a lowered activity of both \(\text{SR Ca}^{2+}\)-ATPase and Na\(^+\)/K\(^+\)-ATPase due to a reduction of their content (20), we also examined these ATP-driven ion pumps in our wild-type and mutant mice. As for human (20), the activity and the concentration of \(\text{SR Ca}^{2+}\)-ATPase as well as the K\(^+\)-dependent, ouabain-sensitive 3-O-MFPase activity and the ouabain-binding capacity, i.e., the number of Na\(^+\)/K\(^+\)-ATPase molecules, are lower in cultured mouse muscle cells than in mouse whole hind limb muscle, but the molecular activities do not differ (Table III). Furthermore, pairwise comparison between the two types of cultured muscle cells or skeletal muscle of wild-type and DMPK\([-/-]\) mice do not reveal any difference in the activity or content of \(\text{SR Ca}^{2+}\)-ATPase and Na\(^+\)/K\(^+\)-ATPase (Table III).

Figure 4. Effects of tetrodotoxin on resting \([\text{Ca}^{2+}]_j\) (A) and depolarization-elicited \(\text{Ca}^{2+}\) responses (B and C) in cultured wild-type and DMPK\([-/-]\) muscle cells. Muscle cells were preincubated with 5 \(\mu\text{M}\) tetrodotoxin for 30 min before depolarization (B and C). For other details, see legend to Fig. 2. Tetrodotoxin partially normalizes the higher resting \([\text{Ca}^{2+}]_j\) of DMPK\([-/-]\) cells (A).


Discussion

The focus of interest in DM research has slowly drifted from the behavior of the unstable (CTG) repeat to the actual function(s) of individual genes in the mutant chromosome 19q area. Three candidate genes for the DM locus have now been characterized: DMR-N9, DMPK, and DMAHP (34, 35). As the expanding repeat in DM actually interrupts the gene for DMPK, most efforts in explaining disease etiology have been directed towards the involvement of the product(s) of this single gene. The results presented in this paper provide novel evidence that there is a specific relationship between DMPK activity and Ca^{2+} homeostasis in skeletal muscle cells. Although results from DMPK-cDNA transfection studies in BCl3H1 cells pointed to a function of DMPK in the myogenic pathway (36), we conclude from study of the close-to-natural context presented here, that the sole absence of DMPK does not appear to interfere with the growth and differentiation profiles of muscle-derived satellite cells in vitro. Neither the morphological appearance, nor the maturation grade, or the ability to contract, differed between wild-type and mutant cells. Moreover, our observations are in accordance with the absence of any gross morphological and structural modifications in vivo in mice that lack or overexpress the DMPK gene (9, 10).

The behavior of Ca^{2+} homeostasis in cultured muscle cells depends on DMPK activity. Estimates for resting [Ca^{2+}] values of mouse skeletal muscle cells in culture clearly depend on the cell origin, the Ca^{2+} indicator used for assaying, and the calibration procedure applied. All we can say is that our value for the resting [Ca^{2+}] in cultured muscle cells of wild-type mice, based on the in vivo calibration of Fura-2, falls well within the range of values published for cultured mouse myotubes (37) and myofibers in vivo (38). Since inhibition of the SR Ca^{2+} release channel by ryanodine effectively blocks depolarization-induced Ca^{2+} efflux in cultured wild-type cells (Fig. 2, B and C), these cells, like those of rats and humans, must possess a skeletal muscle type of E-C coupling mechanism, rather than a cardiac muscle type, in which Ca^{2+} release is provoked by an influx of external Ca^{2+} (28, 31). Moreover, it suggests that the contribution of a Ca^{2+} influx through the ion-unselective AChR (39), the dihydropyridine-insensitive fast-activated voltage-operated T-type Ca^{2+} channel (40), and/or the slow-activated voltage-dependent L-type Ca^{2+} channel, i.e., DHPR (41), during depolarization is negligibly small, and/or is ineffective to induce Ca^{2+} release from the SR. This does not rule out that Ca^{2+} entry via the DHPR may play a role in long-lasting depolarizations in wild-type cells, but usually this process is slower than the charge movement in DHPR and the associated activation of the RyR (41).

The situation in mutant myotubes is clearly different in that absence of DMPK augments the resting [Ca^{2+}], and has dampening effects on the release and sequestration of Ca^{2+} upon depolarization. Application of inhibitors showed that the phenomenon of higher resting [Ca^{2+}], and the altered excitability are somehow linked to the mode of action of voltage-dependent Ca^{2+} and Na^{+} channels. It is unlikely that a recently described Ca^{2+}-specific leak channel is involved, as the situation.
is completely normalized by nifedipine, which fails to inhibit this leak channel (42). Our mutant myotubes lack a protein kinase that is closely related to the subfamilies of cAMP-dependent protein kinases and protein kinases C (2, 43). Hence, it is most likely that the phosphorylation status of the Ca\(^{2+}\) and/or Na\(^+\) channels or any other protein that mediates the clustering of ion channels in the sarclemma is modified in DMPK\([-/-]\) cells. Perhaps more relevant is that re- nal regulation of TTX-sensitive Na\(^+\) channels and voltage-oper- ated L-type Ca\(^{2+}\) channels may have a differential threshold effects due to the completely distinct mutation type in the human and mouse situation. For example, for DM patients it is conceivable that large expansions of the (CTG\(^n\)) repeat in the immediate vicinity, like the down- stream-located DMAHP gene which specifies a transcription factor implemented in ion-channel expression regulation (35, 59).

Thus, although the cell model is clearly too simplistic, our finding of similar disturbances in Ca\(^{2+}\) homeostasis in muscle cells of both DM patients and DMPK\([-/-]\) mice lends indirect support to the contention that the DM mutation results in a reduction or redistribution of DMPK mRNA and protein isoforms, in line with the molecular explanations provided by others (8, 60, 61). In conclusion, our study provides new insight in the relation between DMPK activity and Ca\(^{2+}\) responsive-

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Muscle (Wild-type)</th>
<th>DMPK([-/-]) (Wild-type)</th>
<th>Cultured muscle cells (Wild-type)</th>
<th>DMPK([-/-]) (Wild-type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR Ca(^{2+})-ATPase activity (mU/mg protein)</td>
<td>76.2±7.4 (5)</td>
<td>75.9±4.5 (3)</td>
<td>13.1±2.3 (8)</td>
<td>13.3±1.8 (4)</td>
</tr>
<tr>
<td>SR Ca(^{2+})-ATPase content (pmol/mg protein)</td>
<td>94.9±8.0 (5)</td>
<td>94.7±5.8 (3)</td>
<td>16.4±2.8 (8)</td>
<td>16.5±2.0 (4)</td>
</tr>
<tr>
<td>Molecular activity (min(^{-1}))</td>
<td>802±14 (5)</td>
<td>801±9 (3)</td>
<td>800±8 (8)</td>
<td>803±13 (4)</td>
</tr>
<tr>
<td>Na(^{+})/K(^{-})-ATPase activity (mU/mg protein)</td>
<td>0.57±0.06 (5)</td>
<td>0.56±0.08 (3)</td>
<td>0.93±0.09 (7)</td>
<td>0.95±0.13 (4)</td>
</tr>
<tr>
<td>Na(^{+})/K(^{-})-ATPase content (pmol/mg protein)</td>
<td>6.3±0.6 (5)</td>
<td>6.2±0.2 (3)</td>
<td>10.1±0.7 (5)</td>
<td>10.4±1.6 (4)</td>
</tr>
<tr>
<td>Molecular activity (min(^{-1}))</td>
<td>91±7 (5)</td>
<td>89±11 (3)</td>
<td>92±8 (5)</td>
<td>91±8 (4)</td>
</tr>
</tbody>
</table>

Table III. SR Ca\(^{2+}\)-ATPase and Na\(^{+}/K\(^{-}\)-ATPase in Muscle and Cultured Muscle Cells of Wild-type and DMPK\([-/-]\) Mice

Values are means±SD of the number of muscles or muscle cell cultures (given between parentheses). The activity and content of SR Ca\(^{2+}\)-ATPase were determined by Ca\(^{2+}\)-dependent ATP hydrolysis and steady-state phosphorylation, respectively. The K\(^{+}\)-dependent, ouabain-sensitive hydrolysis of 3-O-MFP and the binding capacity of ouabain were used as a measure for the activity and content of Na\(^{+}/K\(^{-}\)-ATPase, respectively.

Relevance for disease etiology? Since [Ca\(^{2+}\)], is a crucial regulator of many physiological processes, elevated [Ca\(^{2+}\)], could potentially have profound consequences in vivo. Can we extrapolate our in vitro findings and relate abnormal Ca\(^{2+}\) homeostasis to disease manifestation in DM patients? We realize that our knockout mouse and cell models do not provide a direct answer to this question, as the effects of CTG expansion on DMPK expression still have not been clearly resolved. The mouse myotubes, however, do provide the ideal test system to study the normal biological significance of DMPK against a well-defined, constant genetic background. Earlier we discussed the apparent lack of an overt phenotype in our animal model (9) in terms of possible adaptation, compensation, or threshold effects, which in mice, with a relatively low muscle workload due to small body weight, often obscure the manifestation of myopathic features (58). Here we study the system in isolation, outside the context of the entire tissue, and it is perhaps more relevant that the elevated resting [Ca\(^{2+}\)], and the effects on release and sequestration of Ca\(^{2+}\) coupled to abnormal regulation of TTX-sensitive Na\(^+\) channels and voltage-operated L-type Ca\(^{2+}\) channels match the data obtained with myotubes derived from DM patients (15–18). There are also differences in that abnormal SR Ca\(^{2+}\)-ATPase and Na\(^{+}/K\(^{-}\)-ATPase levels were not found in mouse DMPK\([-/-]\) cells (Table III, 20). It is possible that Ca\(^{2+}\)-dependent gene expression critical for these changes may have a differential threshold sensitivity in man and mouse, and almost certainly there are differential effects due to the completely distinct mutation type in the human and mouse situation. For example, for DM patients it is conceivable that large expansions of the (CTG\(^n\)) repeat affect other genes in the immediate vicinity, like the down- stream-located DMAHP gene which specifies a transcription factor implemented in ion-channel expression regulation (35, 59).
ness in skeletal muscle. The suggestion that DMPK modulates the movement of gating charge and/or the activity of voltage-gated ion channels opens up new possibilities for studies into the molecular etiology of DM.

Acknowledgments

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References


