Abstract

Myotonic dystrophy (DM), the most prevalent muscular disorder in adults, is caused by (CTG)_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 [Ca^{2+}], the amplitude and shape of depolarization-induced Ca^{2+} transients, and the content of ATP-driven ion pumps in cultured skeletal muscle cells of wild-type and DMPK[−/−] knockout mice.

In vitro-differentiated DMPK[−/−] myotubes exhibit a higher resting [Ca^{2+}], than do wild-type myotubes because of an altered open probability of voltage-dependent L-type Ca^{2+} and Na^{+} channels. The mutant myotubes exhibit smaller and slower Ca^{2+} responses upon triggering by acetylcholine or high external K+. In addition, we observed that these Ca^{2+} transients partially result from an influx of extracellular Ca^{2+} through the L-type Ca^{2+} channel. Neither the content nor the activity of Na^{+}/K^{+} ATPase and sarcoplasmic reticulum 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 • ion channels • ion pumps • knockout mouse • 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, sensorineu-ral deafness, cardiac conduction defects, hypoversomnia, 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[−/−] 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 fibers and/or cultured skeletal muscle cells of DM patients exhibit a decreased resting membrane potential (11–13) and increased basal cytosolic Na^{+} and Ca^{2+} concentrations (14–16). These features may be attributed to anomalies in the functioning of voltage-operated Na^{+} channels (17, 18) and Ca^{2+} channels (15, 16) and/or to a reduced content of Na^{+}/K^{+}-ATPase and sarcoplasmic reticulum (SR) Ca^{2+}-ATPase. In addition, the persistence of an apamin-receptor, i.e. Ca^{2+}-activated K^{+} channel, has been demonstrated (21).

Many of these ion channels and ion pumps are key players in the excitation-contraction (E-C) coupling mechanism of skeletal muscle in vivo, a cascade of events in which, sequentially, acetylcholine receptors (AChR) of the neuromuscular junction, sarcolemmal tetrodotoxin-sensitive voltage-operated Na^{+} channels (TTXR), T-tubular dihydropyridine receptors (DHPR), and finally ryanodine receptors (RyR), i.e. Ca^{2+} release channels in the terminal cisternae of the SR, are involved (22). E-C coupling of vertebrate skeletal muscle is thought to occur by a mechanical coupling, in which intramembrane charge movements and associated conformational changes of the voltage-sensing DHPR activate the RyR without entry of external Ca^{2+} through DHPR (23–25). Before relaxation, the disturbed ion concentrations are restored to resting levels by ATP-driven ion pumps, i.e., Na^{+}/K^{+}-ATPase and SR Ca^{2+}-ATPase.

This whole process is subject to complex regulation in which local luminal and cytosolic Ca^{2+} and ATP levels are in-
Parameter 6.6 ±1.9*
 Cá II m, 7.9±2.8*
 511 + 124*
 3.2±0.7 3.0±0.6
 8.4 ±2.1*
 Cá II basal controls.

Muscle cells. DMPK activity and ion homeostasis in skeletal muscle. Our finding of abnormally operat­
sequences at the cellular level. Our finding of abnormally operat­

Methods
Materials. The acetoxymethyl ester of Fura-2 (Fura-2/AM) was purchased from Molecular Probes Europe (Leiden, The Netherlands); acetylcholine chloride (ACh), tetrodotoxin (TTX), cyanidine and ionomycin were from Sigma (St. Louis, MO, USA); and nifedipine from Bayer (Leverkusen, Germany). Sources of other materials were described previously (20, 26).

DMPK-deficient mice. Homozygous DMPK-deficient (DMPK[-/-]) mice were generated by targeted mutagenesis and genotyped by PCR analysis as described (9). DMPK[-/-] animals from the F3 generation (on a mixed C57BL/6 x 129/OLA 50-50% background) were used. Wild-type animals from the C57BL/6 inbred strain, and from a 129/OLA x C57BL/6 background (1:2) were used. Wild-type animals from the F3 genera­

Results
General characteristics of cultured wild-type and DMPK[-/-] skeletal muscle cells. Upon differentiation, in vitro skeletal muscle cells derived from wild-type and DMPK-deficient mice exhibit a similar morphological appearance. Typically, the di­

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+]i, max</td>
<td>122±17</td>
<td>185±24a</td>
</tr>
<tr>
<td>20 µM ACh</td>
<td>[Ca2+]i, max</td>
<td>775±131</td>
<td>511±124a</td>
</tr>
<tr>
<td>Ti</td>
<td>3.0±0.6</td>
<td>6.6±1.9a</td>
<td></td>
</tr>
<tr>
<td>Td</td>
<td>4.6±0.8</td>
<td>8.4±2.1a</td>
<td></td>
</tr>
<tr>
<td>125 mM KCl</td>
<td>[Ca2+]i, max</td>
<td>881±164</td>
<td>552±130a</td>
</tr>
<tr>
<td>Ti</td>
<td>3.2±0.7</td>
<td>6.1±1.7a</td>
<td></td>
</tr>
<tr>
<td>Td</td>
<td>4.8±0.7</td>
<td>7.9±2.8a</td>
<td></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 values of [Ca2+]i 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.

Myotonic Dystrophy Protein Kinase Modulates Ca2+ Homeostasis in Muscle 1441
Figure 1. Depolarization-induced Ca\textsuperscript{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\textsuperscript{2+}], after superimposing all appropriate experiments as listed in Table I.

The rest of the text discusses the effects of depolarization and various inhibitors on Ca\textsuperscript{2+} homeostasis in cultured skeletal muscle cells. It mentions the role of tetrodotoxin (TTX) in blocking ACh-evoked Ca\textsuperscript{2+} transients and the effect of Ryanodine on Ca\textsuperscript{2+} levels in wild-type and DMPK[−/−] muscle cells. The text also highlights the differences in Ca\textsuperscript{2+} transients between wild-type and DMPK[−/−] cells, with reduced responses and altered kinetics.

The text further describes the role of DHPRs and TTXRs in Ca\textsuperscript{2+} release and the effects of nifedipine and TTX on these processes. The study concludes with observations on the constitutive open state of DHPRs and TTXRs in resting mutant skeletal muscle cells and the role of Ryanodine and Tetrodotoxin in regulating Ca\textsuperscript{2+} transients.

Figure 2. Effects of ryanodine on resting [Ca\textsuperscript{2+}], (A) and depolarization-provoked Ca\textsuperscript{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\textsuperscript{2+}], after superimposing all relevant experiments as given in Table II. Ryanodine does not normalize the higher resting [Ca\textsuperscript{2+}] of DMPK[−/−] cells (A), and only partially inhibits depolarization-induced Ca\textsuperscript{2+} transients (B and C).
Figure 3. Effects of nifedipine on resting [Ca\(^{2+}\)]\(_i\) (A) and depolarization-generated Ca\(^{2+}\) transients (B and C) in cultured wild-type and DMPK\([-/-]\) muscle cells. Muscle cells were incubated with 5 \(\mu\)M nifedipine for 30 min before depolarization (B and C). For other details, see legend to Fig. 2. Nifedipine completely normalizes the higher resting [Ca\(^{2+}\)]\(_i\) of DMPK\([-/-]\) cells (A).

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

Myotonic Dystrophy Protein Kinase Modulates Ca\(^{2+}\) Homeostasis in Muscle

SR Ca\(^{2+}\)-ATPase and Na\(^+\)/K\(^+-\)ATPase. The here observed 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 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 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 SR Ca\(^{2+}\)-ATPase and Na\(^+\)/K\(^+-\)ATPase (Table III).
The focus of interest in DM research has slowly drifted from the behavior of the instable (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 BC3H1 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$^{2+}$]$_j$, 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$^{2+}$]$_j$ 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$^{2+}$]$_j$, 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 CaV and/or NaV channels or any other protein that mediates the clustering of ion channels in the sarcolemma is modified in DMPK[-/-] myotubes. In turn, this may change the gating properties of these channels and/or influence the distribution and regulation of voltage-dependent SR CaV release events (44). Importantly, phosphorylation of both the α and β subunits of the DHPR is known to occur. Phosphorylation of the α subunit leads to an increase of the CaV current (45), and we would expect absence of phosphorylation to reduce this current, a feature that is inconsistent with the observed high [Ca\(^{2+}\)]j in resting DMPK[-/-] cells. Perhaps more relevant is that recombination of the α subunit, which regulates the α1-subunit activity (47), are unknown. Similarly interesting is that phosphorylation of the α subunit of the TTX-sensitive NaV channel in cultured mouse and rat muscle cells by protein kinase C and in transfected oocytes by DMPK leads to a reduction of the NaV current (48, 49). A persistent influx of NaV as a consequence of DMPK absence may lead to mild long-lasting depolarization of the sarcolemma, and results in a more frequent opening of the L-type CaV channel (46). 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).

**Relevance for disease etiology?** Since [Ca\(^{2+}\)]j is a crucial regulator of many physiological processes, elevated [Ca\(^{2+}\)]j 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 CaV coupled to abnormal regulation of TTX-sensitive NaV channels and voltage-operated L-type CaV channels match the data obtained with myotubes derived from DM patients (15–18). There are also differences in that abnormal SR CaV-ATPase and NaV/KV-ATPase levels were not found in mouse DMPK[-/-] cells (Table III, 20). It is possible that CaV-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 downstream-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 CaV 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 CaV responsive-

---

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

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Wild-type</th>
<th>DMPK[-/-]</th>
<th>Wild-type</th>
<th>DMPK[-/-]</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 (3)</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±0.5 (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 (8)</td>
<td>91±8 (4)</td>
</tr>
</tbody>
</table>

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.
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

The authors are grateful to Dr. T.H. van Kuppevelt for helpful discussions.

This study was supported by grants from the Prinses Beatrix Fonds, the American Muscle Dystrophy Association (MDA), the Association Francaise contre Les Myopathies (AFM) and the Netherlands Organisation of Scientific Research (NWO).

References


Myotonic Dystrophy Protein Kinase Modulates Ca²⁺ Homeostasis in Muscle 1447