Abnormal contractile activity and calcium cycling in cardiac myocytes isolated from dmpk knockout mice

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1Division of Molecular Genetics, Faculty of Biomedical and Life Sciences, Anderson College, University of Glasgow 56 Dumbarton Road, Glasgow G11 6NU; and 2Division of Neuroscience and Biomedical Systems, Faculty of Biomedical and Life Sciences, West Medical Building, University of Glasgow, University Avenue, Glasgow G12 8QQ, Scotland, United Kingdom
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Pall, Gurman S., Keith J. Johnson, and Godfrey L. Smith. Abnormal contractile activity and calcium cycling in cardiac myocytes isolated from dmpk knockout mice. Physiol Genomics 13: 139–146, 2003. First published February 20, 2003; 10.1152/physiolgenomics.00107.2002.—Dysfunction of the gene encoding DMPK (myotonic dystrophy protein kinase) has been implicated in the human neuromuscular disease myotonic dystrophy (DM1). The cardiac features of the disease include progressive conduction defects and ventricular arrhythmias. These defects have been observed in hearts of mice deficient for DMPK function. We have investigated the role of DMPK in the function of ventricular cardiomyocytes using dmpk knockout (KO) mice. A deficit in DMPK caused enhanced basal contractility of single cardiomyocytes and an associated increase in intracellular Ca2+ concentration in mouse ventricular cardiomyocytes. Concurrent with hyperphosphorylated PLB, the response to isoprenaline was reduced. These observations suggest dmpk has a modulatory role in the control of intracellular Ca2+ concentration in mouse ventricular cardiomyocytes, loss of which may contribute to cardiac dysfunction in DM1.

myotonic muscular dystrophy; protein kinase; transgenic model; cardiac defect

MYOTONIC DYSTROPHY type 1 (DM1) is an autosomal dominant neuromuscular disease and is the commonest cause of adult muscular dystrophy. The main clinical indications of the disease include myotonia (the inability to relax a contracted muscle), muscle atrophy, progressive muscle weakness, male infertility, and cardiac conduction defects (14, 15). The primary clinical cardiac manifestation in DM1 is the development of conduction disturbances with progressive atrioventricular (AV) block and ventricular arrhythmias, including tachycardia and bradycardia (3, 10). First degree (prolonged) AV conduction block is common in DM1. Higher grade AV conduction block and ventricular tachycardia are associated with “sudden death” in DM1 (10, 29).

The genetic basis of the disease is the expansion of an unstable triplet CTG repeat on human chromosome 19q13.3 (DM1 locus). The triplet repeat is in the 3'-untranslated region (3'-UTR) region of the DMPK (myotonic dystrophy protein kinase) gene and in the promoter region of the SIX5 gene (formerly known as DMAHP). The link between the genetic and cellular pathology of DM1 is complex but it is clear that this disease is not simply due to the expression of the expanded repeat and that the flanking genes are involved (45, 50). Mouse models deficient in dmpk and SIX5 replicate features of DM1 that include cardiac conduction defects, muscle wasting, cataracts, and gonadal atrophy (3, 16, 20, 38, 40). However, no single mutant alone replicates the severity of the clinical symptoms of DM1 including the triplet expansion mutants (30, 41).

DMPK encodes a serine-threonine protein kinase related to protein kinases C and A, and the Rho family of kinases, which are associated with actin cytoskeleton organization and altered sensitivity of Ca2+ desensitization activated force (21, 35, 45, 51). Uncertainty remains as to the intracellular location of DMPK, evidence exists to suggest that the enzyme is localized to the sarcoplasmic reticulum (SR) and intercalated discs of the heart and in neuromuscular junctions in skeletal muscle (34, 48). Tissue-specific isoforms of DMPK produced from alternatively spliced transcripts partly explain the different expression patterns documented (12). The role of the DMPK protein in vivo in normal cellular processes is not fully understood. However, the generation of mouse mutants deficient in dmpk have revealed altered Na+ ion channel activity and Ca2+ and Na+ ion homeostasis in muscle similar to what is found in DM1 patients (2, 11, 33). Putative substrates for DMPK include myogenin, myosin light chain, muscle Na+ channel, and β-subunit of the L-type Ca2+ channel (6, 46).

To test whether DMPK plays a role in modulating excitation-contraction (E-C) coupling in cardiac myocytes that may be contributory to the cardiac pathology.
observed in DM1, we have studied the contractile behavior of single cardiac myocytes isolated from dmpk knockout (KO) mice. We have employed cell length shortening, intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) measurements, and the response of these cells to the β-adrenergic agonist isoproterenol at a range of stimulus frequencies to characterize the behavior of dmpk KO cells.

**MATERIAL AND METHOD**

**Dmpk KO mice.** The dmpk KO mouse model has been described previously by Jansen et al. (16). A homozygous line established from this transgenic was used to generate male mice for this study.

**Cardiac myocyte isolation.** Single viable cardiac myocytes were isolated from mouse heart using a protocol adapted from published techniques (32). Hearts were dissected from 10-wk-old C57BL/6 wild-type (WT) and dmpk KO male mice and placed in ice-cold Ca\(^{2+}\)-free HEPES-buffered Krebs (HKB) containing 5 U/ml heparin. Hearts were squeezed gently to remove any blood and then cannulated to allow Langendorff perfusion with Ca\(^{2+}\)-free HKB prewarmed at 37°C. Perfusion was at a constant flow rate of 1.5 ml/min for 10 min followed by digestion with 0.67 mg/ml collagenase type I (Worthington) and 0.04 mg/ml protease (Sigma) for 5–7 min. Thereafter, hearts were removed from the perfusion apparatus, atria were discarded, and ventricles were cut into small pieces and resuspended in 10 ml of HKB containing 0.1 mg/ml BSA and triturated to dissociate into single cardiac myocytes. This cell suspension was filtered through a nylon mesh (300 μm) and spun at 2,000 rpm. The resulting soft pellet was resuspended in 10 ml HKB and taken through a gradient of Ca\(^{2+}\)-free HKB to a final concentration of 1 mM Ca\(^{2+}\). Subsequently, cell numbers were determined and cell dimensions (length and width) of 50 myocytes per heart were measured using a hemocytometer and graduated microscope eyepiece. On average 20–25% of cells isolated from WT and dmpk KO hearts were viable rod-shaped cardiac myocytes with intact membranes. Myocytes were stored at room temperature prior to experimentation and used within 4 h after isolation to avoid any deterioration.

**Contractile measurements.** Isolated cardiac myocytes were placed in a cell chamber at 37°C on an inverted microscope stage (Nikon). Myocytes were allowed to settle onto the glass coverslip forming the bottom of the cell chamber and then continuously perfused with HKB containing 1.8 mM Ca\(^{2+}\). The flow of perfusate through the chamber was gravity regulated at 1 ml/min, and the level was kept constant through continuous aspiration. Myocytes to be used for recordings were selected according to the following criteria: rod-shaped appearance with clean edges, clear striations, and no membrane blebs; no spontaneous shortening activity when unstimulated in perfusate containing 1.8 mM Ca\(^{2+}\); steady diastolic length and contractile amplitude at 1-Hz stimulation. Myocytes were electrically stimulated to contract via platinum field electrodes placed in the cell chamber that were attached to a stimulator (Digitimer DS1). The stimulation frequency was progressively increased from 0.3 to 8 Hz. Each rate was maintained until steady-state shortening was reached before taking any recordings. Cell shortening was measured using a video photodiode edge detection system (Cairn Research) with a spatial resolution of 0.65 μm (× 40 objective) and time resolution of 4 ms. The signals were digitized and stored using an IBM computer plus LabPC+ interface analog-to-digital converter (VCAN software developed by Dr. John Dempster, Strathclyde University).

**Measurement of intracellular Ca\(^{2+}\) and SR Ca\(^{2+}\) content.** Intracellular [Ca\(^{2+}\)] was measured from fura-2 fluorescence signals using a dual-wavelength spectrophotometer (Cairn Research). Cytosolic loading of fura-2 was achieved by incubating cardiac myocytes isolated from WT and dmpk KO mice with 5 μM fura-2/AM at room temperature for 10 min. Separate measurements of fura-2 fluorescence ratio (340 nm/380 nm) before and after intracellular equilibration using Ca\(^{2+}\) ionophore solution established a reliable measure of minimum ratio (R\(_{\text{min}}\)) and maximum ratio (R\(_{\text{max}}\)). The values of R\(_{\text{min}}\) and R\(_{\text{max}}\) were not significantly different in cardiomyocytes from different hearts or from the different experimental groups. These fluorescence ratio recordings were converted to Ca\(^{2+}\) concentrations using the conversion factor K\(_A\)((R\(_{\text{max}}\)−R)/R\(_{\text{min}}\)) assuming an association constant of (K\(_A\)) of 1.2 μM, a value determined by equilibrating cardiomyocytes perfused with Ca\(^{2+}\)-ionophores in a range of buffered [Ca\(^{2+}\)]. The SR Ca\(^{2+}\) content was assessed by establishing a steady-state Ca\(^{2+}\) transient at the desired rate and rapidly (1–15 s) stopping stimulation and perfusing with 10 mM caffeine. Caffeine causes a rapid and complete release of Ca\(^{2+}\) from the SR, the ratio was then plotted against the caffeine-induced Ca\(^{2+}\) release is used an indication of SR Ca\(^{2+}\) content.

**Response to isoproterenol.** Isolated WT and dmpk KO cardiac myocytes were perfused with HKB containing 1.8 mM Ca\(^{2+}\) and increasing concentrations of isoproterenol (a β-adrenergic agonist) while being stimulated at 1 Hz at 37°C. Cell length shortening and kinetic parameters were measured as before at 10⁻⁵, 10⁻⁴, and 10⁻³ M isoproterenol. Isoproterenol was prepared fresh for each experiment and protocols were completed within 4 h. Cells were initially selected according to the criteria described above. Following the development of a steady-state shortening on stimulation at 1 Hz, the perfusate was changed to base Krebs containing 10⁻⁸ M isoproterenol and subsequently to higher concentrations of isoproterenol. Measurements were taken after cells had reached a steady state in each concentration. Exposure to isoproterenol could be reversed by perfusion for 10–15 min with HKB containing 1.8 mM Ca\(^{2+}\).

**Statistical analysis.** Data are reported as means ± SE. Statistical comparisons were made with two-tailed Student’s t-test between data collected from different frequency stimulations within control and transgenic groups and also between control and transgenic groups. Measurements of myocyte contractility were calculated for each heart from the average of at least three myocytes. Statistical comparisons between WT and dmpk KO animals were assessed on the number of hearts, and P values of < 0.05 were considered statistically significant.

**Protein analysis.** Cardiac homogenates were prepared from 10-wk-old C57BL/6 WT and dmpk KO male mice. Homogenates were used in preference to isolated cells to monitor accurate changes in phosphorylation status. Studies in our laboratory and others indicate isolated cells, a mix of nonviable hypercontracted and viable striated, display a nonphysiological hyperphosphorylated state of proteins (31). The whole heart was quickly removed and rinsed in ice-cold Krebs solution to remove any blood. The atria and any connective tissue were trimmed from the heart prior to homogenization. The ventricles were placed in 1 ml of ice-cold homogenization buffer [containing 0.3 M sucrose, 10 mM imidazole, pH 7.0, 60 mM histidine, 1 μM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 100 μg/ml aprotinin] and cut into small pieces followed by homogenization using an electrical homogenizer (Kontes, Anachem). The homogenization buffer contained a cocktail of kinase and phosphatase inhibitors (20 μM microcystein, 1

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whole hearts dissected from WT and
myocyte length and width measured for both WT and
significantly different (Table 1). The ranges of
weights of whole hearts and cell dimensions.

RESULTS

Heart weights and myocyte dimensions. Weights of
whole hearts dissected from WT and dmpk KO animals
were not significantly different (Table 1). The ranges of
myocyte length and width measured for both WT and
dmpk KO cells show normal distribution. No signifi-
cant difference in mean cell dimensions was observed,
indicating no cellular hypertrophy present in dmpk KO
animals (Table 1). Myocyte dimensions are comparable
to other species, e.g., rabbit (32). No morphological
differences were observed between cells isolated from
WT and KO hearts under the light microscope (data
not shown).

Myocyte contractility. Cardiac myocytes isolated
from WT and dmpk KO mice were stimulated at fre-
quencies ranging from 0.3 to 8 Hz. This range of fre-
cuencies was used to allow comparison of this study to
previous studies from our laboratory and others. Cell
shortening records (average of 8 sweeps) were mea-
sured for a range of stimulation frequencies. Figure 1
shows the change in diastolic length as frequency of
stimulation is increased. On average, a 10% decrease
in diastolic length is observed both in WT and dmpk
KO cells. For each stimulation frequency, cell length
shortening relative to diastolic length (rCLS) and ki-
etic parameters including time-to-peak shortening
(Tpeak), duration at 50% peak shortening (dur50), and
decay to 90% peak shortening (decay90) were mea-
sured (see inset, Fig. 1).

In WT cells a 2.5-fold increase in rCLS was observed
as stimulation frequency was increased from 0.3 to 8

\[
\begin{array}{cccc}
\text{dmpk KO} & \text{WT} & \text{KO} & \text{WT} \\
\text{Animals} & 10 & 9 & 12 \\
\text{Cells} & 200 & 180 & 220 \\
\end{array}
\]

Values are means ± SE. KO, knockout.

<table>
<thead>
<tr>
<th>Myocyte Dimensions</th>
<th>Organ-to-Body Wt Ratio (×10⁻³)</th>
<th>Heart Wt, mg</th>
<th>Body Wt, g</th>
<th>Cardiac Myocytes</th>
<th>Cell length, µm</th>
<th>Cell width, µm</th>
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<td></td>
<td></td>
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<tr>
<td>WT</td>
<td></td>
<td>221 ± 18</td>
<td>30.4 ± 1.2</td>
<td>7.3 ± 0.5</td>
<td>137.2 ± 2.3</td>
<td>28.6 ± 0.6</td>
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<tr>
<td>dmpk KO</td>
<td></td>
<td>217 ± 12</td>
<td>30.8 ± 0.6</td>
<td>7.1 ± 0.4</td>
<td>135.0 ± 2.4</td>
<td>26.3 ± 0.7</td>
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Inset: representative shortening trace with the kinetic parameters measured to characterize the contractile response: Tpeak, time to peak amplitude; dur50, duration at 50% peak amplitude; decay90, decay to 90% peak amplitude; dia, diastolic cell length; sys, systolic cell length.

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Hz (Fig. 2A). The kinetic parameters measured showed a trend to increased rate of rise and rate of decline as stimulation frequency was increased (Fig. 2B).

Cells isolated from dmpk KO mice showed a significant 2.5-fold increase in rCLS at all frequencies of stimulation (Fig. 2A). The increase in rCLS with increasing stimulation frequency in dmpk KO cells is in parallel with the increase observed in WT cells over the range of stimulation frequencies.

Kinetic parameters in dmpk KO cells showed decreasing profiles with increase in stimulation frequency similar to WT cells. However, compared with WT cells, a shift was observed in the value of each parameter at each frequency measured from dmpk KO cells: on average, Tpeak increased by 16% (Fig. 2A); dur50 and decay90 were both observed to be decreased by 11% and 22%, respectively (data not shown).

Measurement of intracellular Ca\textsuperscript{2+} within cardiac myocyte. Figure 3 shows levels of Ca\textsuperscript{2+} recorded in WT and dmpk KO cells during diastole and systole. In WT cells the intracellular Ca\textsuperscript{2+} levels during diastole and systole increased with increasing frequency of stimulation (Fig. 3). The increased systolic [Ca\textsuperscript{2+}] is consistent with the increase observed in rCLS over the range of stimulation frequencies (Fig. 2A). In the dmpk KO cells (Fig. 3) a similar trend in peak systolic [Ca\textsuperscript{2+}] was observed with increase in frequency. No significant difference in diastolic levels is observed between WT and dmpk KO cells. However, the systolic levels of intracellular Ca\textsuperscript{2+} are significantly higher in dmpk KO cells. Caffeine-induced Ca\textsuperscript{2+} release from the SR was also significantly higher in the dmpk KO cells in parallel with increased peak systolic [Ca\textsuperscript{2+}] and cell length shortening at all frequencies of stimulation (Fig. 4). This suggests that SR Ca\textsuperscript{2+} content was significantly higher at all stimulation frequencies in myocytes isolated from dmpk KO animals.

Response of myocytes to isoprenaline. Increasing the concentration of isoprenaline from 10\textsuperscript{-8} to 10\textsuperscript{-6} M resulted in a fourfold increase in rCLS in WT cells (Fig. 5). Consistent changes in kinetic parameters of the cells were observed (data not shown). Dmpk KO cells exposed to the same increase in isoprenaline concentration displayed a significantly reduced, threefold less, increase in the shortening amplitude (P < 0.05). Parallel changes were observed in the kinetic parameters measured from dmpk KO cells (data not shown).

Protein analysis. Analysis of protein isolated from dmpk KO mice cardiac ventricular muscle shows hyperphosphorylation of PLB (Fig. 6). Immunodetection with the A1 antibody detects PLB in all states and shows higher states of PLB phosphorylation as a mobility shift in the band indicating PLB in the dmpk KO sample. Phosphorylation of both the serine-16 and threonine-17 residues was confirmed by detection with the phosphopeptide-specific anti-
bodies PS16 and PT-17 in WT and dmpk KO (Fig. 6). The levels of SERCA2a were normalized using densitometry against protein stained with Coomassie to show equal loading (data not shown). No significant change in SERCA2a expression was detected (Fig. 6).

DISCUSSION

The results of this study suggest that DMPK plays a modulatory role in E-C coupling in the mouse heart. A deficit in DMPK function leads to abnormal cardiac myocyte contractility and abnormal Ca\(^{2+}\)/H\(^{100}\) cycling. These observations implicate DMPK function as a factor contributing to the cardiac complications observed in DM1 patients.

The changes in cell length and kinetics of cell length shortening indicate altered cardiac contractility in cardiac myocytes isolated from dmpk KO mice. The increased Ca\(^{2+}\) levels in the dmpk KO cells indicate increased Ca\(^{2+}\)/H\(^{100}\) cycling through the SR. This is consistent with the hyperphosphorylated status of PLB in the dmpk KO animals. Increased phosphorylation of PLB results in increased SERCA2a activity and increased SR Ca\(^{2+}\) content. These events are part of the response of mammalian myocardium to \(\beta\)-adrenergic stimulation.

The measurements in myocytes isolated from WT indicate an increase in amplitude of the intracellular Ca\(^{2+}\) transient along with increased myocyte shortening as the frequency of stimulation increased. This indicates that cardiac myocytes isolated from the mouse follow a typical positive force-frequency relationship as observed for other mammalian species (4). This relationship seems not to be altered by the loss of DMPK function. However, at all frequencies of stimulation, peak systolic [Ca\(^{2+}\)] was significantly higher in cardiomyocytes isolated from dmpk KO animals. This suggests that increased intracellular [Ca\(^{2+}\)] is responsible for the increased fractional shortening in the dmpk KO group as opposed to increased myofilament responsiveness (Fig. 2A).
The triplet expansion associated with DM1 in the 3'-UTR of the DMPK gene has been shown to affect gene expression at several levels (15, 45, 50). Studies have shown altered splicing of the DMPK transcript, and it has been demonstrated that the expanded repeat leads to retention of the DMPK mRNA within the nucleus (7, 37, 44, 47). The net effect of abnormal processing of the DMPK transcript is that the level of DMPK is reduced in the disease state (9, 49). Mouse models deficient in DMPK function have been shown to reproduce some of the features of DM1 (16, 38). The cardiac conduction defects observed in these animals show high similarity to the human condition. Studies have suggested that DMPK may mediate effects on cardiac muscle by altering intercellular communication at the intercalated disc (3). However, this study uses single cardiac myocytes and thus eliminates intercellular effects and focuses on the intracellular role of DMPK.

Expression studies, although controversial due to potential cross-reactivity of antibodies, have described DMPK localization in cardiac muscle SR (8, 25, 28, 34, 36, 48). Studies on skeletal muscle isolated from dmpk KO mice have implicated DMPK in the modulation of Ca$^{2+}$ homeostasis potentially through the altered activity of Na$^+$ channels (2, 33).

Contraction of cardiac cells is initiated by the release of Ca$^{2+}$ stored in the SR. The Ca$^{2+}$ is cycled back to the SR to achieve relaxation of the muscle (4). Inability to relax is a primary defect in myotonic muscle, and the rate of relaxation is related to the rate of uptake of intracellular Ca$^{2+}$ by the SR and/or the properties of the myofilaments. Uptake of Ca$^{2+}$ into the SR is determined by the activity of SERCA2a that is regulated by the phosphoprotein PLB (23, 42, 43). Hypophosphorylated PLB inhibits the Ca$^{2+}$ binding activity of SERCA2a; upon phosphorylation PLB dissociates from SERCA2a, and inhibition is released. In vivo PLB functions as a pentamer; each monomer has two sites (serine-16 and threonine-17) which can be phosphorylated by protein kinase A (PKA) or Ca$^{2+}$/calmodulin-dependent protein kinase (CaMKII). The importance of PLB in regulating basal contractility, in mediating the adrenergic response, and in determining the cardiac muscle force-frequency relationship has been established with the use of mouse models deficient in and overexpressing PLB (5, 18, 19, 26, 52). Intracellular Ca$^{2+}$ homeostasis is regulated through the activity of the Na$^+$/Ca$^{2+}$ exchanger (4, 39). Our results support a modulatory role for DMPK in Ca$^{2+}$ handling by the SR.

We have detected hyperphosphorylation of PLB in dmpk KO tissue indicating that DMPK although not regulating PLB directly, may influence the phosphorylation status of PLB. The loss of DMPK results in the increased phosphorylation of PLB; thus we infer DMPK may be involved in negative regulation of the PLB phosphorylation pathway. It may be involved in the inhibition of a pathway leading to phosphorylation of PLB, or alternatively it may activate a phosphatase specific for PLB dephosphorylation in the WT. Kinases, such as Rho, with highest homology to DMPK have been shown to be involved in cell-signaling pathways that regulate the cellular cytoskeleton (13, 51, 22). The hyperphosphorylation of PLB is consistent with the increased basal myocyte shortening in cardiac myocyte contractility and in the dampened response to β-adrenergic agonist isoprenaline. Similar changes in basal contractility and response to β-adrenergic stimulation have been reported in PLB KO mice (27). The consequences of upregulation of SERCA2a activity are an increased SR content and increased propensity to spontaneous Ca$^{2+}$ release (1). Increased SR Ca$^{2+}$ content is known to promote the generation of arrhythmic current in cardiac muscle (17); therefore, the changes observed in the dmpk KO mouse would be expected to have pro-arrhythmic consequences. However, as mentioned previously, the cardiac phenotype in DM1 consists of arrhythmias associated with the conduction system and bradycardia (3); neither condition can be easily related to the findings of this study. In summary, this study provides insight into the role of dmpk in modulating E-C coupling in mammalian cardiac muscle and suggests an additional aspect to the cardiac phenotype that may be present in DM1.

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