MYOTONIC DYSTROPHY PROTEIN KINASE (DMPK) PHOSPHORYLATES PHOSPHOLAMBAN AND REGULATES CALCIUM UPTAKE IN CARDIOMYOCYTE SARCOPLASMIC RETICULUM

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Running Title: DMPK phosphorylates PLN

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Myotonic dystrophy (DM) is caused by a CTG expansion in the 3'-untranslated region of a protein kinase gene (DMPK). Cardiovascular disease is one of the most prevalent causes of death in DM patients and electrophysiological studies in cardiac muscles from DM patients and from DMPK knocked-out mice suggested that DMPK is critical to the modulation of cardiac contractility and to the maintenance of proper cardiac conduction activity. However, there are no data regarding the molecular signalling pathways involved in DM heart failure. Here, we show that DMPK expression in cardiac myocytes is highly enriched in the sarcoplasmic reticulum (SR) where it colocalizes with the ryanodine receptor and phospholamban (PLN), a muscle-specific SR Ca\(^{2+}\) ATPase (SERCA2a) inhibitor. Co-immunoprecipitation studies showed that DMPK and PLN can physically associate. Further, purified wild-type DMPK, but not a kinase-deficient mutant (K110A DMPK), phosphorylates PLN in vitro. Subsequent studies using the DMPK knocked-out mice demonstrated that PLN is hypo-phosphorylated in SR vesicles from DMPK knocked-out mice compared to wild-type mice both in vitro and in vivo. Finally, we show that Ca\(^{2+}\) uptake in SR is impaired in ventricular homogenates from DMPK knocked-out mice. Together, our data suggest the existence of a novel regulatory DMPK pathway for cardiac contractility and provide a molecular mechanism for DM heart pathology.

Introduction

Myotonic muscular dystrophy (DM) is an autosomal, dominant inherited, neuromuscular disorder with an incidence of 1 in 8000 in European and North American populations. Clinical expression of DM is extremely variable; patients present with progressive muscular dystrophy associated with the inability to promote normal muscle relaxation (myotonia), cataracts, cardiac arrhythmia, testicular atrophy, and insulin resistance [1].

The DM1 mutation has been identified as the expansion of an unstable CTG-repeat in the 3'-untranslated region of a gene encoding myotonic dystrophy protein kinase (DMPK), at chromosome 19q13.3 and the age of onset and the severity of the disease correlates with the extent of expansion [2, 3]. The dmpk gene product is a Ser/Thr protein kinase homologous to the MRCK p21-activated kinases [4] and the Rho family of kinases [5]. Data obtained using antibodies that detect specific isoforms of DMPK indicate that the most abundant isoform of DMPK is an 80 kDa protein expressed almost exclusively in smooth, skeletal, and cardiac muscles [6] and this kinase exists both as a membrane-associated and as a soluble form in human left ventricular samples [7]. The different C-termini of DMPK that arise from alternative splicing determine its localization to endoplasmic reticulum, mitochondria, or cytosol in transfected COS-1 cells [8]. Among the substrates for DMPK proposed by in vitro studies are phospholemman, the dihydropyridine receptor and the myosin phosphatase targeting subunit [9-11]. However, an in vivo demonstration of the phosphorylation of these substrates by DMPK remains to be established, and a link between these substrates and the clinical manifestations of DM is unclear. DMPK knock-out mice (DMPK knocked-out) display a cardiac phenotype which reproduces many cardiac conduction defects observed in DM patients, including first-, second- and third-degree A-V block [12-14]. A cardiac phenotype is also…
observed in heterozygous DMPK \(^{+/+}\) mice, which develop first-degree heart block, a conduction defect strikingly similar to that observed in DM patients [15]. DMPK is involved in the modulation of calcium homeostasis in skeletal muscle [16-19] and, in [15] cardiac myocytes isolated from DMPK \(^{-/-}\) mice, enhanced basal contractility and increased intracellular \(\text{Ca}^{2+}\) concentration has been described [20]. However, the exact molecular mechanisms involved in the onset of heart failure in DM patients remain elusive[16-19].

In the heart, calcium cycling across the SR plays a key role in regulating contraction-relaxation cycles [21]. When active, SR \(\text{Ca}^{2+}\)-ATPase (SERCA2a) sequesters cytoplasmic \(\text{Ca}^{2+}\), initiating cardiac relaxation. The activation of SERCA2a is tightly controlled by the SR membrane protein phospholamban (PLN) [22] and chronic PLN-SERCA2a interaction is the critical \(\text{Ca}^{2+}\) cycling defect in dilated cardiomyopathy [23]. Cardiac phospholamban is a 52-amino acid phosphoprotein located in SR membranes with two adjacent residues, Ser16 and Thr17, identified as the phosphorylation sites for PKA and CaMKII, respectively [24-26]. Unphosphorylated PLN inhibits SR \(\text{Ca}^{2+}\) ATPase, whereas phosphorylation of PLN reverses this inhibition [26]. A previous report suggested that PLN was hyperphosphorylated in cardiac homogenates obtained from DMPK\(^{-/-}\) compared to control animals [20]. However, the authors used specific anti-PSer16-PLN and anti-PThr17-PLN antibodies and showed a difference in mobility rather than the intensity of the bands, indicating that the modification of PLN that they observed in DMPK\(^{-/-}\) samples was not Ser16 or Thr17 phosphorylation.

Here, we report that DMPK is highly enriched in cardiac myocyte sarcoplasmic reticulum (SR) and it co-localizes, interacts with and phosphorylates PLN. We demonstrate that PLN is underphosphorylated in SR vesicles from DMPK\(^{-/-}\) mice compared to wild-type mice both \textit{in vitro} and \textit{in vivo}. Finally, we show that SR \(\text{Ca}^{2+}\) uptake is highly reduced in ventricular homogenates from DMPK\(^{-/-}\) mice. The loss of DMPK phosphorylation of PLN may therefore represent an important determinant of ventricular dysfunction in DM.

**Experimental Procedures**

**Materials.** Mouse full length DMPK cDNA was kindly provided by Dr. B. Wieringa (University of Nijmegen, The Netherlands). Human DMPK cDNA subcloned into pRSETc vector was a kind gift from Dr. L. T. Timchenko (Baylor College of Medicine, Houston) and myc-tagged hDMPK was kindly provided by Dr. M. B. Perryman (University of Colorado Health Sciences Center, Denver, CO).

Monoclonal antibodies against human DMPK (MANDM5, 3D10) were provided by Dr. G. E. Morris (MRIC Biochemistry Group, North East Wales Institute, UK). Anti-flag M2 monoclonal antibodies were from Sigma. Anti-phospholamban (clone A1) and anti-phospho-phospholamban (Ser16) were from Upstate (Lake Placid, NY, USA). To detect mouse DMPK we used the polyclonal anti-DMPK antibody from Zymed (San Francisco, CA, USA). Anti-calsequestrin and anti-SERCA2aATPase antibodies were from Affinity Bioreagents (Golden, CO, USA). Monoclonal anti-myc antibody 9E10 was obtained from ATCC (USA).

**Preparation of SR vesicles from mouse left ventricle.** Left ventricles were homogenized in ice-cold solution 1 (10 mM NaHCO\(_3\), 0.2 mM CaCl\(_2\), and protease inhibitors) (1.5 ml/ventricle) by 3 bursts of 10 sec in a polytron-homogenizer at half maximum speed. The homogenate was diluted in an equal volume of ice cold solution 2 (500 mM sucrose, 300 mM KCl, 4 mM MgCl\(_2\), 60 mM histidine pH 7.4) and centrifuged at 1000 \(\times\)g for 20 min at 4°C. The supernatant was recovered (total extract), diluted with 0.25 volumes of 3 M KCl and centrifuged at 10,000 \(\times\)g for 20 min at 4°C. The supernatant was recovered and centrifuged at 100,000 \(\times\)g for 30 min at 4°C. The supernatant was recovered as cytosolic fraction. The pellet was washed in solution 3 (250 mM sucrose, 600 mM KCl, 3 mM MgCl\(_2\), 30 mM histidine pH 7.4) and centrifuged at 100,000 \(\times\)g for 30 min at 4°C. The pellet was finally resuspended in 200 \(\mu\)l of solution 4 (250 mM sucrose, 30 mM histidine pH 7.4) and repeatedly passed through a 25 gauge needle before storage at -80°C. Proteins were measured by the method of Bradford [27].

**Recombinant adenovirus generation and neonatal myocyte gene transfer.** Recombinant adenoviruses expressing myc-tagged human DMPK were generated by homologous recombination as described by Graham and Prevec [28]. The cDNAs were cloned into the shuttle plasmid pAdl1/RSV and co-
transfected with pJM17 into 293 cells to achieve homologous recombination as described previously [29]. Neonatal rat ventricular myocytes were prepared from hearts of 2-3-day-old Sprague-Dawley rat pups as described previously [30]. Briefly, hearts were digested with collagenase and myocytes purified over a Percoll gradient. Myocytes were seeded on coverslips in 4:1 Dulbecco’s modified Eagle’s medium:medium 199 containing 10% horse serum, 5% fetal calf serum, and antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin). Ten hours after isolation and culture, myocytes were infected for two hours with either myc-hDMPK (ad-myc-hDMPK) or green fluorescent protein (ad-GFP) as a control (data not shown) at a multiplicity of infection of 100, before the addition of a suitable volume of culture media. Twenty-four hours later, the myocytes were washed and harvested for immunostaining.

Immunofluorescence and in situ hybridization. Cardiomyocytes plated on coverslips were fixed for 20 min with 3% paraformaldehyde in PBS, washed three times in PBS and then, treated as previously described [31]. Images were obtained using a Leica TCS 4D laser confocal fluorescence microscope with a 40 x objective. For in situ hybridization, seven micron paraffin sections from mouse day 14 embryos were rehydrated, treated with 10mg/ml Proteinase K for 7 min 30 sec and hybridized with 10,000 cpm 35S riboprobe/ml in hybridization solution containing 50% formamide, 30 mM, NaCl, 20 mM EDTA, 10 mM NaH2PO4, 10% dextran sulfate, 1x Denhardt’s and 10mM Dithiothreitol (DTT). Hybridization was carried out for 14 h at 60°C. Samples were subsequently washed at 60°C in 5xSSC/10mM DTT, 2XSSC/50% formamide/10mM DTT; digested with RNase A (10µg/ml) for 30 min at 37°C and dehydrated. The antisense RNA probe was transcribed in vitro at 37°C for 2 h with T7 RNA polymerase after the subcloned plasmid was linearized with Not I. After in vitro transcription the plasmid template was digested with RNase-free DNase. The probe was purified using an RNase free S-200 microspin column (Pharmacia). Slides were dipped in LM-1 photographic emulsion (Amersham Biosciences UK Limited), exposed for ten days and devepbped at 12°C in Kodak D19 solution for 3min 30 sec. Counterstaining was performed in 0.02% toluidin blue and slides were mounted in Permount solution. Images were taken using an Olympus microscope equipped with dark field condenser [32].

Transfections in HeLa cells. Transient transfections were performed in HeLa cells by standard calcium phosphate precipitation in 10 cm diameter plates with a mixture of DNA containing 20 µg of total DNA [31]. The GFP-encoding plasmid was included to monitor transfection efficiencies, which ranged from 60 to 90% as assessed by FACS analysis.

Generation and purification of (His)6 fusion proteins. (His)6-hDMPK cDNA cloned into pRSETc vector (Invitrogen) was used as template to generate the K110A mutant hDMPK with the QuickChange site-directed mutagenesis kit (Stratagene), according to the manufacturer's protocol. The mutagenic oligonucleotide was 5′-CCAGGGATATCCGCTGAGTTTG/3′ (sense strand, the mutated nucleotides 1521 and 1522 are indicated by parentheses). Proper construction of the mutated cDNA was confirmed by complete sequencing. Expression of fusion proteins in Escherichia coli (BL 21) was induced by adding 0.1 mM isopropylthiogalactoside (IPTG) for 3 h to the bacterial culture in the exponential phase of growth. The bacteria were pelleted, resuspended in PBS (pH 7.4), lysed by sonication and pelleted to remove debris. The soluble proteins were purified using chelating Sepharose charged with Ni2+ ions (Amersham Pharmacia Biotech) and visualized by silver staining. Kinase activity of the recombinant proteins was measured as described below by using 10 µg of the purified fractions.

Electrophoresis and immunoblotting. Cells were lysed for 30 min at 4°C in 50 mM Tris pH 7.5, 120 mM NaCl, 1 mM EDTA, 6 mM EGTA, 15 mM Na4P2O7, 20 mM NaF, 0.1% PMSF, 0.1% aprotinin, 2 mM pepstatin, 2 mM leupeptin, supplemented with 1% Nonidet-P40. Cell extracts were centrifuged at 10,000 xg for 20 min at 4°C and 50 µg of the solubilized proteins was loaded. SDS-PAGE and immunoblot analysis were performed as described previously [33]. Protein expression was quantified by scanning densitometry of at least 3 independent experiments for each condition.

Coimmunoprecipitation assays. HeLa cells were cotransfected with myc-hDMPK and flag-PLN. 24
hours after transfection, cells were washed twice in PBS, scraped and solubilized as described above. The supernatants (500 µg) were immunoprecipitated for 90 min at 4°C with anti-myc, anti-flag or non-immune control antibodies preadsorbed on protein-G-Sepharose. The immunopellets were washed 3 times in solubilization buffer before being resuspended in SDS-PAGE sample buffer under reduction conditions and analysed by Western blot using polyclonal antibodies against myc or flag, as indicated.

**Immunoprecipitation and protein kinase activity.** Cell lysates were immunoprecipitated for 2 h at 4°C with protein G-bound anti-myc monoclonal antibody. Immunopellets were rinsed 3 times in lysis buffer and once in kinase buffer (25 mM Tris-HCl pH 8.0, 1 mM EDTA, 10 mM MgCl₂, 5 mM β-glycerophosphate, 2.5 mM DTT). Assays for protein kinase activity were carried out for 1 h at 37°C in a volume of 40 µl of kinase buffer containing 0.1 µM ATP. Substrates used were flag- or myc-tagged PLN expressed in HeLa cells and immunoprecipitated with anti-flag or anti-myc antibodies, respectively. Reactions were stopped with Laemmli sample buffer and samples were loaded onto 15% acrylamide SDS gelsSR vesicles (10 µg) were solubilized for 30 min at 4°C with 1% Nonidet-P40 in the presence of protease inhibitors and phosphorylation was carried out in the kinase buffer described above for 30 min at 37°C, using 0.1 µM [γ-³²P]ATP (1.5 mCi/µmol) (Amersham, England) and in the presence of 5 µM PKA peptide inhibitor, PKI (Calbiochem, UK). Gels were dried and developed by autoradiography. Phosphorylation was quantified by scanning densitometry of three independent experiments.

**Measurements of SR Ca²⁺ uptake.** Ventricular tissue from 3-4 months old DMPK⁺/⁺ and DMPK⁻/⁻ mouse hearts was homogenized at 4°C in 2.5 ml of homogenizing solution (25 mM imidazole, pH 7.0) with a Teflon glass Thomas tissue grinder. SR Ca²⁺ uptake assays were performed in ventricular homogenates at room temperature based on a protocol modified from that of Pagani and Solaro [34]. Aliquots (175 µl) of homogenates were transferred into tubes containing 1.4 ml of uptake buffer (100 mM KCl, 10 mM K-oxalate, 40 mM imidazole, 10 mM sodium azide, 4. mM MgCl₂, 1 µM Ruthenium Red) and ⁴⁵Ca-EGTA buffer containing 0.185 µCi/ml ⁴⁵Ca (Perkin Elmer) and a given amount of free Ca²⁺ (20 or 200 nM), which was calculated on the basis of the amount of added EGTA. Ruthenium Red was used to block Ca²⁺ efflux via the ryanodine receptor. After 5 min of preincubation, the uptake reaction was initiated by the addition of 2.5 mM sodium ATP. Ca²⁺ uptake was terminated at various time (1, 3, and 5 min for 200 nM free Ca²⁺; 1, 10, and 20 min for 20 nM free Ca²⁺) by filtering 250 µl aliquotes on 0.45 µm nitrocellulose membranes (Millipore-type MA), followed by two washes (5 ml) with uptake buffer without Ca²⁺ and ATP. The remaining radioactivity on the nitrocellulose filters was determined by liquid scintillation spectroscopy. Protein concentration was assayed with a DC protein assay kit (BioRad). Ca²⁺ uptake was calculated from the slope of the linear regression analysis relating ⁴⁵Ca²⁺ uptake/milligram of protein to reaction time.

**Results**

**Expression pattern and cellular distribution of DMPK**

DMPK under-expression contributes to DM pathology, however little is known regarding the specific cell types in which it is expressed. To obtain specific single cell resolution data on dmpk expression, we have performed radioactive in situ hybridization and immunohistochemistry analysis. In the developing mouse embryo, dmpk mRNA is primarily detected in striated muscle structures that include all major muscles in the skeletal structures (Fig. 1A, B), cardiac muscle, and diaphragm (Fig 1D). We also detected dmpk mRNA in the smooth muscle of the lung and gut. We do not detect dmpk expression in the epithelium of the gut at this developmental stage (Fig. 1C). In the heart, dmpk expression is restricted to the cardiomyocytes in the ventricle and atrium, and we do not detect a hybridization signal in the epicardium nor in the endocardium. In the myocardium, the dmpk hybridization signal is stronger in the compact muscular zone compared to the trabecular zone and the hybridization signal is almost indistinguishable from background in the primordia of the papillary muscles. We also observed dmpk expression in the muscular portion of the outflow tract (Fig 1D).
Previous work suggested that DMPK associates with the sarcoplasmic reticulum and gap junctions in rat cardiac muscle [35]. However, those immunocytochemistry and electron microscopy data need to be re-defined, because they were obtained using a polyclonal antibody that detected a 54 kDa band by Western blot, while it is now known that the main DMPK cardiac isoform is an 80 kDa protein [35]. Accordingly, we subsequently performed immunofluorescence studies to characterize the subcellular distribution of DMPK in cardiomyocytes, using a specific anti-hDMPK monoclonal antibody [6]. In adult human cardiomyocytes (Fig. 2 A-C), DMPK specifically co-localized with the ryanodine receptor (RyR), which has been characterized by ultrastructural studies as mainly confined to the SR membranes [36]. Moreover, forced expression of myc-tagged hDMPK by adenoviral gene transfer to rat neonatal cardiomyocytes, showed that myc-hDMPK was targeted to perinuclear structures where it colocalized with the SR-proteins RyR and phospholamban (PLN) (Fig. 2 D-L). Control experiments with adenoviruses expressing recombinant GFP, exhibited both cytosolic and nuclear localization (data not shown), but do not co-localize with ER markers.

As an approach to estimate the percentage of DMPK localized in the SR, we performed subcellular fractionation of mouse left ventricles and analyzed by Western blot the proteins obtained at different steps in the fractionation procedure (10 µg of total extract, cytosol and SR-enriched low density membranes). Taking into account the relative abundance of DMPK in each fraction and the total amount of protein recovered in each fraction, we determined that 31 ± 4 % (n=3) of total left ventricle DMPK localizes to the low-density microsomal fraction. This fraction, although enriched in SR membranes, contains other subcellular membranes where DMPK may also be expressed. (Fig. 2M). Interestingly, DMPK is distributed similarly to the SR protein PLN (Figure 2M, lower panel).

**DMPK interacts with and phosphorylates PLN.**

Since DMPK seems to be highly abundant in the SR of cardiomyocytes (Fig. 2M), co-localizes with phospholamban in these cells (Fig. 2 A-L), and the phospholamban sequence contains several putative DMPK-phosphorylation sites [7, 8], we hypothesized that PLN could be a substrate for DMPK. We generated a hDMPK mutated at the ATP binding site (K110A), which is a kinase deficient mutant as observed using MBP as a substrate (data not shown). We purified bacterially-expressed recombinant (His)_6-hDMPK (WT) and a (His)_6-(K110A)hDMPK (K110A). We observed that flag-PLN expressed in HeLa cells and immunoprecipitated with anti-flag antibody was phosphorylated at Ser16 by wild-type but not by K110A-hDMPK (Fig. 3A). To rule out any effect due to improper folding of bacterially expressed DMPK, we also measured the kinase activity of myc-hDMPK expressed in HeLa cells, observing the phosphorylation of PLN at Ser16 by myc-DMPK (Fig. 3B). No specific phosphorylation on PLN Thr17 was detected under our experimental conditions (data not shown).

DMPK and PLN physically interact, as shown by coimmunoprecipitation studies (Fig. 4). HeLa cells cotransfected with myc-DMPK and flag-PLN were coimmunoprecipitated with either anti-myc (Fig 4A) or anti-flag (Fig. 4B) antibodies to immunoprecipitate DMPK or PLN, respectively, or control non-immune mouse IgGs. The association of DMPK and PLN was clearly evident in both conditions by assess flag-PLN in the myc-DMPK immunopellet and vice versa.

**Decreased PLN phosphorylation activity in left ventricle SR vesicles from DMPK−/− mice.**

We analyzed the phosphorylation state of PLN in SR vesicles purified from 12-week-old male wt and DMPK −/− mice. In both groups of animals, we examined the relative abundance of P-Ser16 PLN and the expression of the SR proteins SERCA2a ATPase, PLN, and calsequestrin. While the steady state levels of all the proteins analysed was similar in SR vesicles from DMPK−/− and wt mice, the amount of P-Ser16 PLN decreased by 1.8±0.2 fold (n=4) in DMPK−/− mice compared to wt animals (Fig. 5A).

As described above (Fig. 3), DMPK phosphorylates PLN in vitro. To analyze whether this reaction occurs in cardiac myocyte SR, we measured the endogenous PLN phosphorylation activity of SR vesicles in the presence of kinase buffer and [γ-32P]ATP in SR vesicles from wt and DMPK−/− mice (Fig. 5B). A strongly phosphorylated band was detected which we identified as PLN considering that it is the only phosphoacceptor protein so far described in cardiomyocytes with a molecular weight of 6 kDa.
PLN phosphorylation was 2.2±0.4 (n=3) fold higher in vesicles from DMPK+/+ animals than that observed in vesicles from DMPK-/- animals. These results provide evidence of a direct link between DMPK expression and PLN phosphorylation state. 

**Decreased Ca\(^{2+}\) uptake in left ventricle SR from DMPK-/- mice.**

We next explored the physiological relevance of our set of data showing (i) localization of DMPK in the SR, (ii) interaction DMPK/PLN and, (iii) DMPK phosphorylation of PLN, by comparing the SR calcium uptake rates in left ventricles from the DMPK-/- and DMPK+/+ mice. Calcium uptake rates were markedly reduced in DMPK-/- mice observing a 48% and 84% decrease at 200 nM and 20 nM free calcium concentrations, respectively, compared to those of controls (Table 1, Fig 6). These results are concomitant with the decrease in PLN phosphorylation in DMPK-/- mice and cannot be attributed to alterations in the content of the SR Ca\(^{2+}\)-cycling proteins SERCA2a and PLN that showed similar expression levels in both DMPK-/- and DMPK+/+ mice (Fig 5, A).

**Discussion**

Cardiovascular disease is one of the most prevalent causes of death in DM patients, accounting for about 30% of fatalities. Cardiac mortality occurs because of progressive left ventricular dysfunction, ischemic heart disease, pulmonary embolism, or unexpected sudden death [37]. Electrophysiological studies in skeletal and cardiac muscles from DM patients and from DMPK-/- mice showed defects in intracellular Ca\(^{2+}\) cycling [16-20]. Here, by means of localization studies, biochemical analysis, and calcium uptake measurements, we describe a novel substrate for DMPK that provides a new mechanism for cardiac dysfunction in DM.

We provide evidence for a preferential muscle-restricted expression of *dmpk* during mouse development. Skeletal and cardiac muscle, as well as smooth muscle in the gut and lung, are positive for *dmpk* signal in mouse embryos. This is in agreement to a recent report describing the expression pattern of *dmpk* in adult tissues [38]. In the developing heart, we did not detect hybridization signal in the epicardium or in the endocardium, suggesting that DMPK is primarily restricted to the contractile muscle. This is of particular importance when addressing cardiomyopathy, since many cardiac phenotypes are consequence of mutation in non-muscular genes [39] and references therein).

Our data also demonstrate that DMPK is enriched in cardiomyocyte SR. Interestingly, following adenoviral gene transfer of myc-tagged DMPK to ventricular cardiomyocytes from neonatal rats, the kinase is targeted to the SR where it co-localizes with PLN, showing a similar pattern of cellular distribution to that observed for the endogenous DMPK in human cardiac myocytes. The observation that DMPK adenoviral gene transfer results in a correct targeting of the kinase to SR suggests that DMPK gene-delivery strategies may become feasible for the improvement of the DM ventricular dysfunction.

The screening of a library of synthetic peptides has shown that the optimal DMPK substrate sequences should comprise three to four arginines (or lysines) at distinct positions N-terminal to the phosphoacceptor [8]. Remarkably, the PLN sequence surrounding Ser16 is similar to that of DMPK preferred Ser-substrate: (R/K)XXXRRf(S)Xf (where X is any amino acid, preferably not P or E, f is a hydrophobic residue and boldface type indicate the phosphoacceptor). In contrast, PLN Thr17 does not fit any of the defined consensus sequences as there is no basic residue at positions -1 or -2 from this Thr, which has been defined as one of the most important features of DMPK phosphorylation on Thr residues. Consistent with these analyses of phosphorylation consensus sequences in synthetic peptides, we demonstrate here that DMPK can phosphorylate PLN at Ser16, while we found no evidence of any specific phosphorylation in threonine 17.

Moreover, in SR vesicles purified from DMPK-/- mice, endogenous PLN 32P-incorporation was 2 fold decreased compared to control animals directly correlating a decreased PLN phosphorylation activity with the lack of DMPK expression. Under physiological conditions, PLN phosphorylation at Ser16 by PKA is the predominant event so far described that leads to proportional increases in the rate of Ca\(^{2+}\) uptake into SR and accelerates ventricular relaxation [40, 41]. DMPK phosphorylation of Ser16-PLN emerges as a novel mechanism involved in the regulation of cardiac contractility that may be therapeutically relevant due to the preferential
muscular expression pattern of DMPK. The proposed model is supported by our data on Ca^{2+} uptake in the DMPK^-/- mouse. A decrease in Ca^{2+} uptake is a central feature of human and animal heart failure and an increase in the amount of PLN associated with SERCA2a is an important determinant of SR dysfunction in the heart [21, 42, 43]. Indeed, the finding that DMPK deficiency results in a marked decrease in SR Ca^{2+} uptake activity points to PLN phosphorylation by DMPK as a physiological relevant event. Importantly, the decrease in SR Ca^{2+} uptake is detectable at 14 weeks of age, which is much earlier than the first detectable signs of muscle weakness (7-10 months) [12] in these animals, thus suggesting that a decrease in SR Ca^{2+} uptake is primary to cardiac dysfunction.

In conclusion, our data indicate that ventricular function can be regulated through DMPK phosphorylation of PLN and provide a molecular mechanism to the ventricular dysfunctions detected in both DM patients and DM animal models. Given that PLN is markedly underphosphorylated in most acquired forms of heart failure, the identification of the molecular signalling pathways involving DMPK in the heart may provide crucial molecular tools to design new therapies for the treatment of DM and other cardiomyopathies.

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Footnotes
The abbreviations used are: DM, myotonic dystrophy; DMPK, myotonic dystrophy protein kinase; PLN, phospholamban; SR, sarcoplasmic reticulum, PKA, CAMP-dependent protein kinase; RyR, ryanodyne receptor

Figure Legends

Figure 1. Expression pattern of DMPK at e14.5 mouse embryos. DMPK mRNA is detected at all major striated muscle tissues that include all skeletal muscle in the back (A) and leg (B). DMPK is also expressed in the smooth muscle of the gut (C) and in the diaphragm and in the heart (D).

Figure 2. Double immunofluorescent staining of DMPK and ryanodine receptor (RyR) in cardiomyocytes. (A-C) Localization of DMPK in adult human myocytes. Simultaneous double-immunofluorescent pictures of DMPK and RyR (merge) show colocalization of both proteins. (D-F) Colocalization of DMPK in neonatal rat cardiomyocytes infected with adenovirus expressing myc-tagged DMPK was analysed by double immunofluorescent staining. Ryanodine receptor (RyR) and PLN were used as marker for sarcoplasmic reticulum and α-actinin was used as a control. Adenovirus expressing GFP were used as a control (data not shown). (M) Subcellular distribution of DMPK in adult rat left ventricles. After subcellular fractionation, relative abundance of DMPK and PLN was analysed in 10 μg from total extracts (total), cytosolic fraction (cytosol) and low-density SR-enriched membranes (SR). Representative images from 3 independent experiments are shown.

Figure 3. DMPK phosphorylates PLN in vitro. (A) Flag-PLN was expressed in HeLa cells, immunoprecipitated with an anti-flag antibody and phosphorylation reaction was carried out in the
presence of 10 µg of bacterially-expressed rhDMPK (WT), rhDMPK(K110A) or a non-specific preparation from non-transformed bacterial extract (NS). PLN-phosphorylation was detected by Western blot using an anti-P-Ser16 PLN antibody. Equivalent amounts of flag-PLN were immunoprecipitated in each condition as observed after re-blotting with an anti-PLN antibody. (B) HeLa cells were transfected with myc-DMPK or myc-PLN and cell extracts from each condition (10 µg or 100 µg, respectively) were independently immunoprecipitated using anti-myc antibody. The immunopellets were combined as indicated in the Figure, and phosphorylation was carried out in the presence of kinase buffer. PLN phosphorylation was detected by western blot using an anti-P-Ser16 PLN antibody (blot: anti-P-Ser16 PLN). The amount of total DMPK immunoprecipitated was analysed with 3D10 monoclonal antibody to hDMPK (blot:DMPK). 10 µg of non-transfected cells (nt) were immunoprecipitated with anti-myc antibody as non-specific control for kinase activity. Data from representative experiments are shown.

**Figure 4. Coimmunoprecipitation of DMPK and PLN.** mycDMPK and flagPLN cDNAs were cotransfected in Hela cells. Cell extracts were immunoprecipitated either with anti-myc, anti-flag, or non-specific immunoglobulins (ns Ig). The pellets were extensively washed and analysed by Western blot using monoclonal antibodies to DMPK (3D10) or to PLN (cA1) to analyse their content in the immunopellets (pt) and the supernatants (snt). Data shown are representative of 3 independent experiments.

**Figure 5. Decreased PLN phosphorylation activity in SR vesicles from DMPK−/− mice.** (a) Left ventricle SR-vesicles were prepared from DMPK+/+ or DMPK−/+ mice. Content of the indicated proteins in 10 µg of SR vesicles was analysed by Western blot. Images shown are representative of 4 independent experiments. (b) Left ventricle SR vesicles from DMPK−/− or DMPK+/+ mice (10 µg) were incubated in a kinase reaction containing [γ-32P]ATP and PKA peptide inhibitor (PKI) for 30 min at 37°C. Reactions were stopped with Laemmli sample buffer and samples were loaded onto 15% acrylamide SDS gels. Gels were dried and visualized by autoradiography. The image shown is representative of 3 independent experiments.

**Table 1 and Figure 6. SR Ca2+ uptake in DMPK+/+ and DMPK−/− mice.** SR Ca2+ uptake assays were performed in ventricular homogenates from DMPK+/+ and DMPK−/− mice at 200 and 20 nM free calcium and at room temperature (n=3). SR Ca2+ uptake (nmol/min/mg protein) was calculated from the slope of the linear regression analysis relating 45Ca2+ uptake per milligram of total protein to reaction time. Activity represent the percentage of DMPK+/+ SR Ca2+ uptake for each free calcium concentration. P<0.01.

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Figure 1

A: bm
B: lg
C: gut
D: ot, gm, d, v, rm, bm, back muscle; lg, leg; gt, gut; d, diaphragm; ot, cardiac outflow tract; v, ventricular muscle; rm, rib muscle.
Figure 2
Figure 3
Figure 4
Figure 5
Table 1. SR Ca\textsuperscript{2+} uptake in DMPK\textsuperscript{+/+} and DMPK\textsuperscript{-/-} mice.

|        | 200 nM                  |           | 20 nM                  |           |
|--------|-------------------------|-----------|-------------------------|-----------|
|        | Ca\textsuperscript{2+} uptake (nmol/min/mg) | activity (%) | Ca\textsuperscript{2+} uptake (nmol/min/mg) | activity (%) |
| DMPK\textsuperscript{+/+} | 107.71±0.47              | 100±0.43  | 8.82±1.11              | 100±12.5  |
| DMPK\textsuperscript{-/-}  | 56.41±5.07               | 52.4±4.7  | 1.38±0.78              | 15.64±8.84 |

![Bar chart showing Ca\textsuperscript{2+} uptake comparison between DMPK\textsuperscript{+/+} and DMPK\textsuperscript{-/-} mice at 200 nM and 20 nM concentrations.](chart.png)
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