Biochemical and Biophysical Research Communications 423 (2012) 212–217

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Regulation of the alternative splicing of *sarcoplasmic reticulum* Ca^{2+}-*ATPase1* (*SERCA1*) by phorbol 12-myristate 13-acetate (PMA) via a PKC pathway

Yimeng Zhao [a], Michinori Koebis [a], Satoshi Suo [a], Shigeo Ohno [b], Shoichi Ishiura [a,*]

[a] Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan
[b] Department of Molecular Biology, Yokohama City University Graduate School of Medical Science, 3-9 Fuku-ura, Kanazawa-ku, Yokohama 236-0004, Japan

ARTICLE INFO

Article history:
Received 6 May 2012
Available online 15 May 2012

Keywords:
Myotonic dystrophy
Sarcoplasmic/endoplasmic reticulum
Ca^{2+}-ATPase 1 (SERCA1)
Alternative splicing
PKC
Phorbol 12-myristate 13-acetate (PMA)
CUG-binding protein 1 (CUGBP1)

ABSTRACT

Myotonic dystrophy type 1 (DM1) is a multi-systemic disease with no established treatment to date. Small, cell-permeable molecules hold the potential to treat DM1. In this study, we investigated the association between protein kinase C (PKC) signaling and splicing of *sarcoplasmic reticulum* Ca^{2+}-*ATPase1* (*SERCA1*). Our aim was to clarify the mechanisms underlying the regulation of alternative splicing, in order to explore new therapeutic strategies for DM1. By assessing the splicing pattern of the endogenous *SERCA1* gene in HEK293 cells, we found that treatment with phorbol 12-myristate 13-acetate (PMA) regulated *SERCA1* splicing. Interestingly, treatment with PMA for 48 h normalized *SERCA1* splicing, while treatment for 1.5 h promoted aberrant splicing. These two responses showed dose dependency and were completely abolished by the PKC inhibitor Ro 31-8220. Furthermore, repression of PKCβII and PKCθ by RNAi mimicked prolonged PMA treatment. These results indicate that PKC signaling is involved in the splicing of *SERCA1* and provide new evidence for a link between alternative splicing and PKC signaling.

1. Introduction

Myotonic dystrophy type 1 (DM1) is an inherited multi-systemic disorder caused by the aberrant expansion of CTG repeats in the *myotonic dystrophy protein kinase* (*DMPK*) 3′-untranslated region (3′-UTR). The clinical presentation of DM1 is highly variable, involving multiple organs, with symptoms including myotonia, cataracts, cardiac conduction defects, progressive muscle wasting and weakness, insulin resistance, and mental retardation. DM1 is a progressive disease; its symptoms become severe with age and across generations.

One reason why aberrantly expanded CTG repeats cause such diverse symptoms is explained by the RNA gain-of-function theory [1]. Expanded CTG repeats are transcribed into RNA with expanded CUG repeats, which possess muscleblind-like protein 1 (MBNL1) binding motifs. MBNL1 is a splicing factor that regulates alternative splicing in several genes (*Clcn1*, *IR*, *cTNT2*, and *SERCA1*) to normalize DM1 splicing abnormalities [2–4]. Ablation of MBNL1 function leads to mis-splicing of several genes. In addition, MBNL1-knockout mice show DM1-like splicing abnormalities and myotonia in their skeletal muscles [5,6]. Moreover, MBNL1-included foci, formed in the nuclei as a result of expanded CUG repeats, were observed by fluorescent immunostaining [3,7]. Thus, levels of free MBNL1 in the cytosol can be decreased by long CUG repeats. Without regulation by MBNL1, the alternative splicing of multiple genes becomes abnormal. Abnormally spliced transcripts are degraded by the nonsense-mediated mRNA decay (NMD) system or translated into abnormal proteins, thereby leading to DM1 symptoms.

Due to the diverse symptoms affecting multiple organs in DM1, its treatment has been limited to supportive care; no basic remedy has been developed. One possible basic remedy is the use of antisense nucleotides to alter the splicing patterns of genes that are aberrantly and indirectly regulated by CUG repeats. However, this method can only treat one gene-derived symptom at a time and requires a great deal of time and effort. Thus, two treatment strategies have been devised: antisense nucleotides against CUG repeats and small molecules. The first strategy uses antisense nucleotides to target CUG repeats and thereby cause repeats to segregate [8], repress [9], or dissolve [10]. However, how to deliver antisense nucleotides into living cells and achieve continual administration remains to be resolved. The other strategy is pharmacological therapy using small molecules. This method can resolve cell permeabilization problems. TG003 has been identified as a compound that improves normal splicing in Duchenne muscular dystrophy (DMD) [11]. In DM1, similarly effective treatment is expected [12,13].

SERCA1 is an aberrantly spliced gene in DM1. SERCA1 protein regulates intracellular Ca^{2+} homeostasis in skeletal muscle cells. The switching of SERCA1 from a fetal isoform, SERCA1b (lacking

Abbreviations: SERCA1, *sarcoplasmic reticulum* Ca^{2+}-*ATPase1*; PMA, phorbol 12-myristate 13-acetate; DM1, myotonic dystrophy type 1; DMPK, myotonic dystrophy protein kinase; MBNL1, muscleblind-like protein 1; CUGBP1, CUG-binding protein 1; CELF, CUG-BP and ETR-3-like (embryonic lethal abnormal vision-type RNA-binding protein 3-like) factor.

* Corresponding author.
E-mail address: cishiura@mail.ecc.u-tokyo.ac.jp (S. Ishiura).

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http://dx.doi.org/10.1016/j.bbrc.2012.05.033

exon 22), to a mature isoform, SERCA1a (which contains exon 22), is thought to play a central role in muscle development. Since exon 22 contains a stop codon, SERCA1b is six amino acid residues longer than SERCA1a. No specific protein structure has been identified in this six-amino acid residue region [14]. However, in normal skeletal muscle tissues, the expression of *SERCA1a* is strictly regulated [4]; *SERCA1b* is only detected in DM1 patients. Other studies have reported that extended, injured, and fetal muscles express *SERCA1b* [15,16]. Determining the differences between the two isoforms of SERCA1 and their functions is important to our understanding of the DM1 pathogenesis. Expression of *SERCA1b* in DM1 patients indicates that there is some deficiency in this pattern-shift point or in myogenesis. MBNL1 has been identified as a splicing factor that regulates the alternative splicing of *SERCA1* and promotes the production of *SERCA1a* [4]. In this report, we attempted to clarify the mechanisms underlying *SERCA1* splicing to determine the pathogenesis of DM1.

Approximately 500 kinds of protein kinases are involved in signal transduction. Collectively, they regulate diverse cell events, including apoptosis, mitosis, and responses to exogenous stimulators. In the present study, we found that prolonged treatment with phorbol 12-myristate 13-acetate (PMA) normalized *SERCA1* splicing. PMA is a well-known protein kinase C (PKC) activator that imitates diacylglycerol (DAG) and binds to the C1A and C1B domains of PKC isozymes. However, prolonged stimulation with PMA downregulates PKC, thereby inhibiting PKC activation.

PKC regulates various cellular processes including apoptosis, cell division, and cell proliferation. This class of serine/threonine-specific protein kinases can be divided into three groups according to basic structure: conventional PKCs (cPKCs), novel PKCs (nPKCs), and atypical PKCs (aPKCs). The cPKCs (PKCα, PKCβI, PKCβII, and PKCγ contain C1A and C1B domains (DAG binding site) and a C2 domain (Ca^{2+} binding site). The nPKCs (PKCδ, PKCε, PKCη, PKCθ, PKCμ, and PKCν) contain only C1A and C1B domains. The aPKCs (PKCξ and PKCι) have no special DAG or Ca^{2+} binding domains. In the normal state, PKC isozymes are usually inactivated by self-inhibitory effects. cPKC isozymes require Ca^{2+}, DAG, and phosphatidyl serine (PS) for activation, while nPKCs are Ca^{2+}-independent and aPKCs can be activated by PS alone.

The association between DM1 pathogenesis and PKC has been described in two reports. CUG-binding protein 1 (CUGBP1) belongs to the CUG-BP and ETR-3-like (embryonic lethal abnormal vision-type RNA-binding protein 3-like) factor (CELF) family and is one of the key factors inducing aberrant splicing in DM1 (*CLC1*, *cTNT* [17] and *Ca(V)1.1* [18]). It is hyperphosphorylated by CUG repeats and promotes an increase in the steady-state level of CUGBP1. In addition, PKC activation is required for this hyperphosphorylation. Specifically, PKCα and PKCβII have been shown to directly activate CUGBP1 *in vitro* [19]. Furthermore, administration of the PKC inhibitor Ro 31-8220 to DM1 model mice ameliorated cardiac conduction defects in DM1 [20].

In this report, we describe a series of experiments that explored the properties of PKC-mediated alternative splicing using the PKC activator PMA and the PKC inhibitor Ro 31-8220. By exploring the mechanisms regulating the splicing of *SERCA1*, we found that PKCβII and PKCθ were involved in the regulation of *SERCA1* splicing. This report identifies a new regulator of *SERCA1* splicing and a therapeutic strategy for DM1.

2. Materials and methods

2.1. PMA treatment and RT-PCR

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and incubated in an atmosphere containing 5% CO_2 at 37 °C. PMA (final concentration, 5–500 nM) was applied for between 5 min and 48 h. Concentrations of PMA higher than 500 nM induced cell death at 48 h.

Cells were washed with 1 × PBS before cultivation. Total cellular RNA was purified using a GenElute Mammalian Total RNA Miniprep kit (Sigma–Aldrich, MO, USA). Next, 2.5 μg of total RNA was reverse transcribed using a PrimeScript 1st Strand cDNA Synthesis kit (TAKARA BIO, Shiga, Japan) and oligo(dT) primers. *SERCA1* was used to assess the compound's ability to alter splicing patterns. Exons 21 to 23 of endogenous *SERCA1* were amplified by PCR using the following primers: forward, 5′-ATC TTC AAG CTC CGG GCC CT-3′; reverse, 5′-CAG CTC TGC CTG AAG ATG TG) [4]. The annealing temperature was 63.5 °C and there were 30 amplification cycles. PCR products were separated by electrophoresis in an 8% polyacrylamide gel, stained with ethidium bromide, and analyzed using an LAS-3000 luminescence image analyzer (Fujifilm, Tokyo, Japan). Band intensities were digitized and quantified using Multi-gauge (Fujifilm, Tokyo, Japan). Exon 22 inclusion rate (*SERCA1a* percentage) was represented as normal band percentage. Statistical analysis was performed using GraphPad Prism 4 (Graphpad Software, CA, USA).

2.2. RNA interference

For RNAi, siRNAs specific for PKC isozymes except PKCα were designed using BLOCK-iT™ RNAi Designer (Invitrogen, CA, USA). PKCα specific siRNA (SIGMA Genosys, Tokyo, Japan) was designed according to [21]. All siRNA (see sense-strand siRNA sequences in Supplementary Table S1) and negative control RNAi (Stealth™ RNAi Negative Control Low GC Duplex #2) were purchased from Invitrogen and transfected with RNAi MAX (Invitrogen, CA, USA) according to the manufacturer's procedure. Cells were cultured for 48 h after transfection. In the case of PMA treatment, PMA (500 nM) was added to the medium 4 h after transfection.

To verify RNA interference, gene-specific primer sets [22–24] were used to amplify endogenous mRNA. Semi-quantitative RT-PCR or quantitative PCR (see primers and amplification conditions in Supplementary Table S2–S4) was performed. Quantitive PCR was performed with Power SYBR Green PCR Master Mix (Applied BioSystems) using a StepOnePlus™ Real Time PCR System (Applied Biosystems, CA, USA) according to the manufacturer's protocol.

3. Results

3.1. PMA regulates SERCA1 exon 22 splicing

To explore the relationship between PKC and *SERCA1* splicing, we first conducted reverse transcription polymerase chain reaction (RT-PCR) analysis to detect changes in splicing induced by PMA. PMA activates PKC when applied for a short period of time and acts as a PKC downregulator when applied for longer periods of time. As PKC is usually in an inactivated (dephosphorylated) state in cells [25], PMA was considered as an idle compound to explore the regulation of alternative splicing by PKC signaling. We assessed the splicing pattern of endogenous *SERCA1*. *SERCA1a* (3570 bp), which is a normal spliced variant, contains exon 22, while the abnormally spliced variant *SERCA1b* (3528 bp) lacks it. Exons 21–23 of *SERCA1* mRNA were amplified (*SERCA1a*, 240 bp; *SERCA1b*, 198 bp) (Fig. 1A). *SERCA1* is aberrantly spliced in DM1 patients and its splicing pattern is strictly regulated in individuals without DM1. This suggests that regulation of the alternative splicing of *SERCA1* may play a central role in the pathogenesis of DM1. However, the mechanism of *SERCA1* splicing remains to be elucidated.

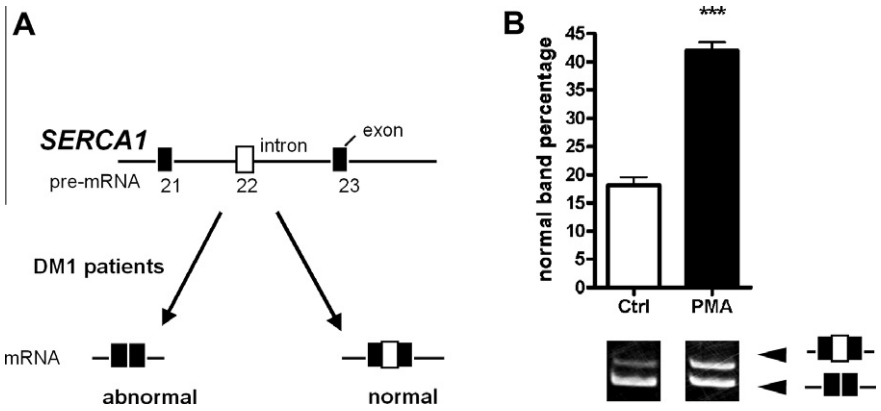

Fig. 1. PMA affects *SERCA1* splicing. (A) Schematic diagram showing two *SERCA1* splicing patterns. (B) RT-PCR analysis of the effects of treatment with 500 nM PMA for 48 h on endogenous *SERCA1* splicing as compared with DMSO (control). Significant differences: $^{***}P < 0.001$ (Student's *t*-test; mean ± SE; $n = 3$).

Applying 500 nM PMA for 48 h to downregulate PKC in HEK293 cells significantly improved *SERCA1a* splicing (Fig. 1B). It was previously shown that the PKC inhibitor Ro 31-8220 normalized DM1 abnormalities [20], suggesting that downregulation of PKC by PMA led to a change in the *SERCA1* splicing pattern.

3.2. Time-course and dose-curve of PMA effects on SERCA1 exon 22 splicing

To further investigate the signaling pathway involved in the regulation of splicing by PMA, we conducted time-course and dose-curve PMA treatment experiments. Since PKC activation by PMA begins within 5 min [26–29], the time points were set to between 5 min and 72 h (5 min and 0.5, 1.5, 3, 10, 24, 48, and 72 h). After 48 h, normal splicing was increased (Fig. 2A). Promotion of normal splicing continued after 72 h. However, aberrant *SERCA1* splicing was significantly increased after 1.5 h (Fig. 2B). These results indicate dual regulation of alternative splicing of *SERCA1* by PMA.

In the dose-curve analysis, the effects of 5, 50, and 500 PMA were compared with a control (no PMA). The effects of PMA on *SERCA1* splicing at both 1.5 and 48 h were dose-dependent (Fig. 2C and D), supporting the idea that PMA regulates *SERCA1* splicing at both time points. In addition, as 500 nM PMA had the strongest effects on *SERCA1* splicing at both 1.5 and 48 h, subsequent PMA experiments were all conducted with a PMA concentration of 500 nM.

Based on these results, we predicted that PKC plays a critical role in *SERCA1* splicing. We hypothesized that PKC signaling regulates alternative splicing, its activation promotes aberrant splicing of *SERCA1*, and its inhibition or downregulation improves normal splicing of *SERCA1*.

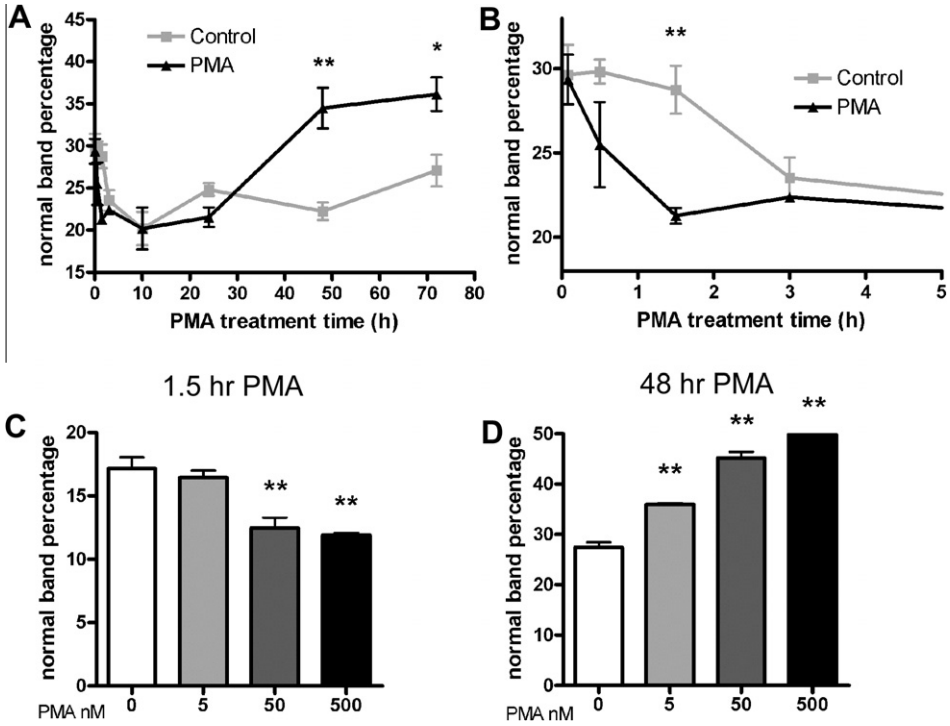

Fig. 2. Regulation of *SERCA1* splicing by PMA. RT-PCR analysis of endogenous *SERCA1* splicing. (A) Time-course of *SERCA1* splicing after stimulation with PMA (500 nM). (B) Data for the first 5 h of the time-course. Controls were treated with DMSO alone. Significant differences: $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's *t* test; mean ± SE; $n = 3$). Dose-curve for the effect of PMA on *SERCA1* splicing (C and D). RT-PCR analysis of normally spliced endogenous *SERCA1* levels in HEK293 cells treated with 0 nM PMA (control) or 5, 50, or 500 nM PMA for 1.5 h (C) or 48 h (D). Significant differences: $^{**}P < 0.01$ (Dunnett's test; mean ± SE; $n = 3$).

3.3. PKC regulates SERCA1 exon 22 splicing

To confirm the involvement of PKC signaling in the response to PMA treatment, the effects of co-treatment with PMA and the PKC inhibitor Ro 31-8220 were examined. Concurrent treatment with Ro 31-8220 (1 μM) completely abolished the effect of PMA treatment (500 nM) for 1.5 h; the level of normally spliced *SERCA1* was upregulated to the same level as in the control (Fig. 3A). Treatment with Ro 31-8220 alone promoted normal *SERCA1* splicing. The effect of PMA (500 nM) at 48 h was completely blocked by Ro 31-8220 (1 μM) (Fig. 3B). Based on these results, we conclude that downregulation of PKC leads to the promotion of *SERCA1a* splicing at 48 h. As 12 PKC isozymes with different functions in different signal transduction pathways have been identified to date, we hypothesized that one or more specific PKC isozymes are involved in the regulation of *SERCA1* splicing.

3.4. Suppression of PKCβII and PKCθ improves SERCA1a splicing

PMA normalizes *SERCA1* splicing, but multiple PKCs (cPKCs and nPKCs) with DAG binding motifs (C1A and C1B sites) can be activated or downregulated (after prolonged stimulation) by PMA. Hence, conventional and novel PKC isozymes may be involved in the regulatory effect of PMA on *SERCA1* splicing. Since aPKCs do not respond to DAG, they can be excluded as candidates. We sought to determine which of the cPKC or nPKC isozymes respond to treatment with PMA for 48 h and improve the production of normal *SERCA1* transcripts. Using RNA interference, we selectively suppressed PKC isozymes and determined whether knockdown of specific isozymes could mimic prolonged PMA stimulation (Fig. 4). Suppression of PKCβII and PKCθ increased normal *SERCA1* splicing, similar to stimulation with PMA for 48 h, while suppression of other PKC isozymes did not affect *SERCA1* splicing. Based on these results, we conclude that depletion of PKCβII and PKCθ improves *SERCA1a* splicing in HEK293 cells.

4. Discussion

In this study, we clarified the mechanism underlying PKC-mediated regulation of *SERCA1* splicing abnormalities in DM1. First, we identified PMA, a widely-used PKC activator and downregulator of PKC during prolonged stimulation, as a compound that effectively normalizes *SERCA1* splicing in HEK293 cells. By examining the properties of PMA's regulatory effects on *SERCA1* splicing, we found that PMA improved normal splicing during treatment for 48 h, while it increased aberrant splicing at 1.5 h. Two peaks for the effects of PMA on *SERCA1* splicing were confirmed to be dose-dependent. We then attempted to determine whether PKC is involved in PMA stimulation using the PKC inhibitor Ro 31-8220. Ro 31-8220 abolished the effects of PMA at both 1.5 and 48 h. However, treatment with Ro 31-8220 alone for 48 h did not affect splicing at all. This may be due to self-inhibition of PKC, meaning that Ro 31-8220 could not inhibit PKC, leaving *SERCA1* splicing unchanged. We thus confirmed the involvement of PKC in the regulation of *SERCA1* splicing by PMA.

The alternative splicing of several genes, including *Bcl-x*, *Axl* [30], and *CD45* [31], has have been reported to be affected by PKC-mediated phosphorylation. In addition, the connection between PKC and splicing-related factors (PSF, hnRNP A3, p68 RNA helicase, and hnRNP L) supports the idea that PKC regulates alternative splicing [32]. However, no specific PKC isozyme has been identified as a splicing regulator.

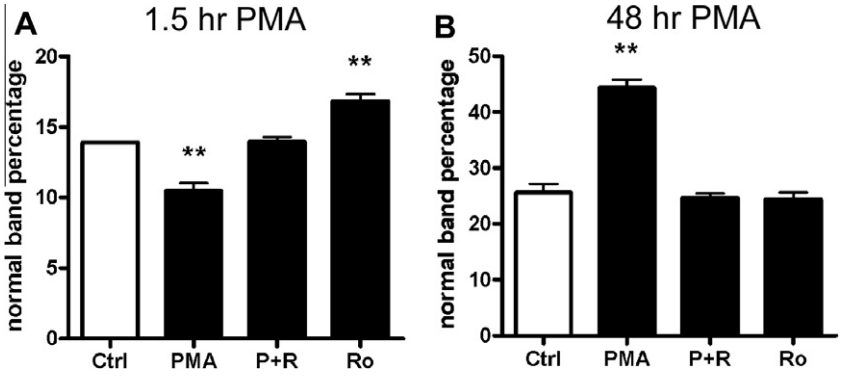

Fig. 3. PMA regulates *SERCA1* splicing via a PKC pathway. RT-PCR analysis of endogenous *SERCA1* splicing in HEK293 cells. PMA and the PKC inhibitor Ro 31-8220 were co-administered for 1.5 h (A) or 48 h (B). Controls were treated with DMSO alone. P = PMA (500 nM); R, Ro = Ro 31-8220 (1 μM). Significant differences: $^{**}P < 0.01$ (Dunnett's test; mean ± SE; $n = 3$).

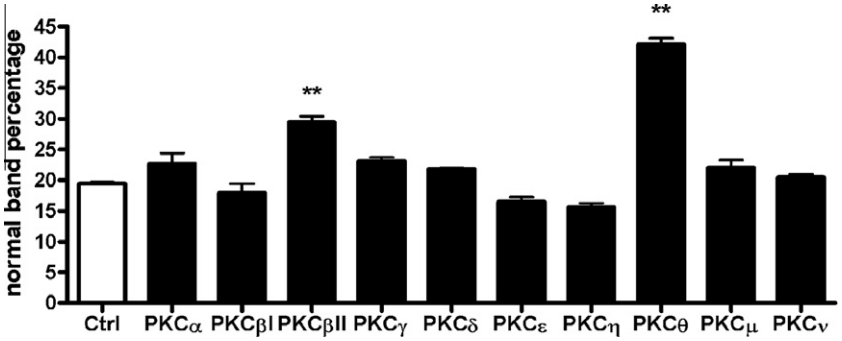

Fig. 4. RNA interference of PKC isozymes mimicked PMA regulation of *SERCA1* splicing. RT-PCR analysis of normally spliced endogenous *SERCA1* in HEK293 cells. RNAi specific for PKC isozymes (PKCα, PKCβI, PKCβII, PKCγ, PKCδ, PKCε, PKCη, PKCθ, PKCμ, or PKCν) as compared with control siRNA. Significant differences: $^{**}P < 0.01$ (Dunnett's test; mean ± SE; $n = 3$).

PKC is known to be involved in many diseases, including cancer, Alzheimer's disease, autoimmune diseases, and cardiovascular diseases. The association between the pathogenesis of DM1 and PKC was mentioned in previous reports [19,20], and CUGBP1 is the key to this association. Hyperphosphorylation of CUGBP1 has been confirmed in DM1 tissues, cells, and model mice and promotes an increase in the steady-state levels of CUGBP1. PKC activation is required for this hyperphosphorylation. Specifically, PKCα and PKCβII have been shown to directly activate CUGBP1 *in vitro* [19]. Furthermore, administration of the PKC inhibitor Ro 31-8220 to DM1 model mice ameliorated cardiac conduction defects [20]. These reports are consistent with our finding that PMA regulates the alternative splicing of *SERCA1* via a PKC pathway.

By selectively reducing the expression of PKC isozymes with siRNA, we identified PKCβII and PKCθ as isozymes that mimic 48 h PMA treatment. These two PKC isozymes promote production of the aberrant isoform *SERCA1b*. PKCθ is mainly expressed in skeletal muscle and T cells, which suggests that it plays crucial roles in myogenesis and the immune system [33,34]. Very recently, abolishing PKCθ in *mdx* (DMD model) mice was shown to prevent muscle wasting and improve muscle regeneration, maintenance, and performance [35]. DMD and DM1 are both progressive muscular dystrophies that have multiple symptoms in common (muscle weakness and muscular atrophy). Similar to DMD, our results showing that reductions in endogenous PKCθ levels in HEK293 cells induced normal *SERCA1* splicing in DM1 suggest that PKCθ is a candidate pharmacological therapeutic target in DM1. PKCβII, a Ca^{2+}-dependent isozyme, was shown to be involved in PMA-mediated CUGBP1 hyperphosphorylation, which induced DM1-like splicing abnormalities in a previous study[20]. So, we hypothesize that CUGBP1 may respond to PMA-induced PKC signaling. Interestingly, however, there were two discrepancies in the response of CUGBP1 to PKC signaling in the regulation of *SERCA1* splicing. First, CUGBP1 was previously shown to have no direct effect on *SERCA1* splicing [4], as demonstrated by overexpression of CUGBP1 in HEK293 cells and deduced from the observation of altered splicing of the *SERCA1* minigene and of endogenous *SERCA1* in C2C12 primary murine myoblast cells. The other difference is that CUGBP1 hyperphosphorylation peaks 3 h after PMA treatment [19]. This is 1.5 h after our *SERCA1* splicing pattern. We are going to check this issue by multiple methods: silencing endogenous CUGBP1 expression and overexpress recombinant CUGBP1, or other CELF families. Unfortunately, the slight but significant effect of stimulation with PMA for 1.5 h prevented us from identifying the PKC isozymes that respond to treatment with PMA for 1.5 h using siRNA.

To summarize, this is the first report to show that PKCβII and PKCθ are involved in the regulation of *SERCA1* alternative splicing. Note that PKCθ has not been reported to be involved in the regulation of alternative splicing. These findings suggest the existence of a neo-alternative splicing regulation pathway that operates via PKC. In conclusion, we not only identified novel potential therapeutic targets for DM1 treatment, but also showed the existence of a new alternative splicing regulatory mechanism.

Acknowledgments

We thank Drs. Y. Kino, K. Kanno, and Y. Nagara for valuable discussions. This work was supported by intramural research Grants (23-5) for Neurological and Psychiatric Disorders from the NCNP, Ministry of Health, Labour and Welfare, Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.033.

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