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Protein kinase D controls voluntary-running-induced skeletal muscle remodelling

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Abstract

Skeletal muscle responds to exercise by activation of signalling pathways that co-ordinate gene expression to sustain muscle performance. MEF2 (myocyte enhancer factor 2)-dependent transcriptional activation of MHC (myosin heavy chain) genes promotes the transformation from fast-twitch into slow-twitch fibres, with MEF2 activity being tightly regulated by interaction with class IIa HDACs (histone deacetylases). PKD (protein kinase D) is known to directly phosphorylate skeletal muscle class IIa HDACs, mediating their nuclear export and thus derepression of MEF2. In the present study, we report the generation of transgenic mice with inducible conditional expression of a dominant-negative PKD1kd (kinase-dead PKD1) protein in skeletal muscle to assess the role of PKD in muscle function. In control mice, long-term voluntary running experiments resulted in a switch from type IIb + IIc/x to type IIa plantaris muscle fibres as measured by indirect immunofluorescence of MHCs isoforms. In mice expressing PKD1kd, this fibre type switch was significantly impaired. These mice exhibited altered muscle fibre composition and decreased running performance compared with control mice. Our findings thus indicate that PKD activity is essential for exercise-induced MEF2-dependent skeletal muscle remodelling *in vivo*.

Keywords

exercise; fibre type switch; muscle remodelling; myocyte enhancer factor 2 (MEF2); myosin heavy chain (MHC); protein kinase D (PKD)

AUTHOR CONTRIBUTION

Kornelia Ellwanger, Christine Kienzle and Sylke Lutz performed the experiments. Zheng-Gen Jin, Maria Wiekowski and Klaus Pfizenmaier participated in the design and proofreading of the paper prior to submission. Angelika Hausser designed the experiments and wrote the paper.

INTRODUCTION

Skeletal muscle is composed of a heterogeneous population of myofibres, which differ in their metabolic and contractile properties and are classified based on their expression of MHC (myosin heavy chain) genes. Type I fibres (slow-twitch fibres) express MHC type I, exert slow contraction, are oxidative and rich in mitochondria and myoglobin, and have a high resistance to fatigue, whereas fast-twitch or type II fibres express MHC type II, exert quick contraction, fatigue rapidly, and rely on glycolytic (type IIb and IIc/x) or oxidative (type IIa) metabolism. Physiological signals such as exercise induce signal transduction pathways that promote adaptive changes in the protein composition and cytoarchitecture of myofibres, thus transforming pre-existing type II fast-twitch fibres into type I slow-twitch fibres [1]. One important transcription factor involved in the regulation of myofibre remodelling is MEF2 (myocyte enhancer factor 2) [1,2]. There is substantial evidence that MEF2 is a key regulator of skeletal muscle development and myofibre remodelling. MEF2 is preferentially activated in slow oxidative fibres to support Ca^{2+} -dependent signalling pathways that promote fibre type remodelling [2]. Moreover, skeletal muscles of MEF2-knockout mice demonstrate a reduction in type I fibres. Conversely, expression of a constitutively active MEF2 protein increases the number of slow-twitch fibres in skeletal muscle and thus exercise endurance and muscle performance [3]. Transcriptional activity of MEF2 is inhibited by its direct interaction with members of the class IIa HDACs (histone deacetylases), which repress the ability of MEF2 to bind DNA [2]. Class IIa HDAC family members include HDAC4, HDAC5, HDAC7 and HDAC9. The activity of class IIa HDACs towards MEF2 is highly controlled by phosphorylation on conserved serine residues. Phosphorylation at these sites induces binding of HDACs to 14-3-3 proteins, thereby mediating unmasking and masking of nuclear export and nuclear localization sequences respectively. The phosphorylation-dependent interaction with 14-3-3 proteins results in the retention of class IIa HDACs in the cytoplasm, the release of MEF2 and thus derepression of downstream target genes [4]. For example, phosphorylation-dependent nuclear export of class IIa HDACs was demonstrated for HDAC5 in cultured myoblasts upon initiation of the muscle differentiation programme [5]. In addition, HDAC4 and HDAC7 translocate from the nucleus to the cytoplasm in a signal-dependent manner in cultured adult skeletal muscle [6,7] and during MEF2-mediated differentiation of mouse myoblasts [8] respectively.

Several serine/threonine kinases can phosphorylate class IIa HDACs at the 14-3-3-binding sites, including CaMK (Ca^{2+} /calmodulin-dependent protein kinase) II [9,10], AMPK (AMP-activated protein kinase) [11], the AMPK family kinase Mark2 [12], and the salt-induced kinase Sik1 [13]. In an elegant screen, Chang and co-workers [12] identified PKD (protein kinase D) as an additional class IIa HDAC kinase. The PKD family of serine/threonine kinases consists of three isoforms: PKD1, PKD2 and PKD3. Previous work has demonstrated a functional interplay between PKD and HDACs in agonist-dependent cardiac hypertrophy. Vega and co-workers [14] have shown that PKD1 directly phosphorylates HDAC5 thereby promoting binding of 14-3-3 proteins and nuclear export. The ability of PKD to phosphorylate and inhibit HDAC5 correlates with pathological remodelling of the heart: expression of PKD1ca (constitutively active PKD1) or excessive activation of PKD1 leads to cardiac remodelling, heart failure and death [15]. Conversely, heart-specific deletion

of PKD1 diminishes hypertrophy and pathological remodelling in response to pressure overload and chronic adrenergic signalling [16]. Interestingly, all four class IIa HDACs contain the PKD substrate sequence, and it was demonstrated that PKD1 is also able to directly phosphorylate HDAC4, HDAC7 and HDAC9 [12,14,17,18]. Besides PKD1, PKD2 and PKD3 are also capable of phosphorylating class IIa HDACs *in vitro* and in monolayer cell cultures [19,20], indicating that, at least with respect to HDAC phosphorylation, the individual PKD isoforms are functionally redundant.

Thus far, relatively little information is available on the functional role of PKD in adult skeletal muscle. Recently, it was demonstrated that α -adrenergic signalling, which is typically active in parallel with motor neuron input during muscular activity, causes the nuclear efflux of HDAC5 in a PKD-dependent manner in cultured slow soleus muscle fibres [7]. Furthermore, Kim and co-workers [21] have reported a role for PKD1 in MEF2-dependent skeletal muscle function. The authors demonstrated that skeletal-muscle-specific overexpression of PKD1ca promotes phosphorylation of HDAC4 and HDAC5, the formation of slow-twitch fibres and an increase in the levels of specific contractile proteins. In addition, skeletal muscle of these mice displayed fatigue resistance in an *ex vivo* muscle contraction model. However, although the skeletal-muscle-specific knockout of PKD1 resulted in increased susceptibility to fatigue, no changes in fibre type composition were observed. To explain these findings it was argued that PKD2 and PKD3 most probably compensate for PKD1 loss [21]. To address whether functional loss of PKD has an impact on skeletal muscle remodelling we therefore generated transgenic mice expressing a PKD1kd (kinase-dead PKD1)–GFP (green fluorescent protein) variant in a conditional and inducible manner under the control of the CMV (cytomegalovirus)/ β -actin promoter. The PKD1kd–GFP protein is known to act in a dominant-negative manner thus inhibiting endogenous PKD signalling [22]. In these mice, doxycycline treatment induced strong PKD1kd–GFP expression predominantly in skeletal muscle. Fluorescence microscopy of cryosections demonstrated uniform PKD1kd–GFP expression with dominant localization of the protein in the nuclei. Voluntary wheel running experiments revealed that running performance of mice expressing the dominant-negative PKD1 variant was significantly decreased compared with control mice. In line with this, analysis of skeletal muscle fibre composition after voluntary wheel running demonstrated that, compared with control animals, mice expressing PKD1kd–GFP contained significantly lower amounts of type IIa fibres, whereas the amount of type IIb and IIc/x fibres was increased. On the basis of our present results, we conclude that expression of dominant-negative PKD1–GFP is sufficient to inhibit MEF2-dependent skeletal muscle remodelling induced by voluntary wheel running, demonstrating for the first time an essential role for PKD in exercise-induced skeletal muscle remodelling.

MATERIALS AND METHODS

Transgenic mice

For the generation of Tg(tetO–PKD1kd–EGFP) mice, the cDNA encoding EGFP (enhanced GFP)-tagged human PKD1kd (K612W) [23] was inserted into the multiple cloning site of pBI-5 [24] via SalI and EcoRV restriction sites. The construct contains a downstream rabbit

β -globin intron/polyadenylation signal. The construct for microinjection containing the tetO, hCMV (human CMV) promoter sequences, PKD1kd-GFP and β -globin was excised from prokaryotic vector sequences via NarI and BsrBI restriction sites (see Figure 1B). Pronucleus microinjection was performed by standard procedures [25]. Tail DNA from founder mice was digested with EcoRV and analysed by Southern blotting with a 577-bp EGFP probe (see Figure 1C). Genotyping was routinely performed by PCR using a primer pair specific for mouse and human PKD1 (forward, 5'-TTGGTCGTGAGAAGAGGTCAAATTC-3'; reverse, 5'-CACCAAGGCAGTTGTTTGGTACTTT-3'). A 246-bp fragment was indicative of transgenic human PKD1kd and a 399-bp fragment of endogenous mouse PKD1 (see Figure 1D). Genomic DNA was obtained from tail tips. PCR was performed in a 20 μ l reaction mixture containing standard buffer and 0.5 μ M of each primer. The cycling conditions consisted of an initial 2-min denaturing step at 95 °C, followed by 36 cycles of 45 s at 95 °C, 45 s at 60°C, and 60 s at 72 °C. Genotyping of rtTA mice was performed as described previously [26].

All animal handling and experiments carried out in the present study were approved by the Regierungspräsidium Stuttgart and complied with local guidelines and regulations for the use of experimental animals (35-9185.81/0209, 35-9185.81/0247).

Conditional expression of PKD1kd-GFP in transgenic mice

To induce transgene expression, double transgenic mice were given a solution of 2 mg/ml doxycycline hyclat (Fagron) and 5 % sucrose in sterile double-distilled water as drinking water. Doxycycline-containing drinking water was protected from light and replaced every 3–4 days. Control animals were either single or non-transgenic animals treated with doxycycline-containing drinking water or double transgenic animals treated with drinking water containing 5 % sucrose without doxycycline.

Antibodies

Commercially available antibodies used were: mouse anti-GFP monoclonal antibody (Roche Applied Science), rabbit anti-CaMKII polyclonal antibody (M-176; Santa Cruz Biotechnology), rabbit anti-PKC (protein kinase C) μ polyclonal antibody (C20; Santa Cruz Biotechnology), rabbit anti-phospho-PKD1 (Ser⁹¹⁶) polyclonal (Cell Signaling Technology), rabbit anti-PKD2 polyclonal antibody (Calbiochem), mouse anti-tubulin- α monoclonal antibody (Ab-2; Neomarkers) and mouse anti-(transferrin receptor) monoclonal antibody (Zymed Laboratories). The mouse monoclonal anti-PKD1 antibody JP2 [27] and the rabbit polyclonal anti-PKD3 antibody [28] have been described previously. Mouse monoclonal antibodies against MHC type I and type IIa were purified from the supernatant of the hybridoma cell lines BAD5 and SC-71 respectively (both from the German Collection of Microorganisms and Cell Cultures), according to the manufacturer's protocol. Fluorescently labelled secondary antibodies for immunofluorescence were Alexa Fluor[®] 546-coupled goat anti-(mouse IgG) (Invitrogen) and NL637-coupled donkey anti-(mouse IgG) (R&D Systems). The secondary IRDye[®]-conjugated antibodies for Western blotting were from LI-COR Biosciences.

Protein extraction from tissue and Western blotting

For Western blot analysis, mouse tissue was homogenized in glass tubes containing 5 μ l of lysis buffer [150 mM NaCl, 20 mM Tris/HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1 % Triton X-100, 1 $\times$ Complete protease inhibitor cocktail (Roche Applied Science), 0.5 mM PMSF, 1 mM sodium fluoride, 1 mM sodium orthovanadate and 20 mM 2-glycerophosphate] per mg of tissue with 15–20 strokes at 800 rev./min of the Potter S homogenizer (Sartorius). Lysates were clarified by centrifugation at 16000 g and 4°C for 15 min. Protein concentrations were determined by the Bradford method using a Bio-Rad Laboratories protein assay solution. Equal amounts of proteins were subjected to SDS/PAGE and blotted on to nitrocellulose membranes (Pall). After blocking with 1 % blocking reagent (Roche Applied Science), membranes were probed with the specific antibodies. Proteins were visualized with IRDye®-coupled secondary antibodies. Quantitative analysis was performed with the Odyssey software (LI-COR Biosciences).

Reporter gene assay

C2C12 cells were plated on to 12-well tissue culture dishes at a density of $\sim 2.0 \times 10^4$ cells/well and were transfected with 100 ng each of the 3xMef2 firefly luciferase reporter plasmid [29], pRL-TK, a *Renilla* luciferase plasmid under the control of the thymidine kinase promoter, and 300 ng of plasmids encoding cDNAs of PKD1wt (wild-type PKD1), PKD1kd or PKD1ca [30] using Lipofectamine™ 2000 (Invitrogen). Cells were harvested 24 h after transfection and luciferase activities were determined as described previously [31]. Acetylcholine (Sigma–Aldrich) was applied at 1 mM for 6 h prior to cell lysis.

In vitro kinase assay

In vitro CaMKII kinase assays were performed as described previously [32] with minor modifications. CaMKII was precipitated from 500 μ g of muscle lysate using 2 μ g of polyclonal anti-CaMKII antibody. Immunocomplexes were precipitated by centrifugation and washed three times with a buffer containing 10 mM Tris/HCl (pH 7.2), 1 mM sodium pyrophosphate and 1 mM EGTA. One-third of each sample was used for the kinase reaction either in the presence or absence of CaCl_2 and to detect CaMKII levels by Western Blot analysis respectively. For the kinase reactions, immunoprecipitated proteins were added to a reaction mixture composed of 10 mM Hepes (pH 7.2), 5 mM MgCl_2 , 1 mM EGTA, 0.1 mM sodium pyrophosphate, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (3000 Ci/mmol), 25 mM auto-camtide-2 substrate, with (maximal) or without (basal) 1.2 mM CaCl_2 and 1.2 mM calmodulin in a final reaction volume of 100 μ l. The reaction proceeded at 30 °C for 15 min and was terminated by spotting 20 μ l of each reaction mixture on to a P81 phosphocellulose filter paper (Whatman). The reaction mixture was absorbed before washing in 75 mM phosphoric acid four times for 10 min each. All reactions were run in duplicate. The incorporation of ATP into the autocamtide-2 peptide was quantified using a PhosphorImager (Storm; Molecular Dynamics).

Voluntary wheel running

To determine voluntary wheel running behaviour, mice were housed individually in type 3 cages (23 cm $\times$ 27 cm $\times$ 43 cm) supplemented with running wheels 20 cm in diameter (Robowheel). Wheel running performance was measured by recording wheel revolutions

continuously with the help of magnetic reed switches. Animals were maintained on a 12/12-h light/dark cycle and provided with standard chow *ad libitum*. Female mice of 5–7 weeks age were used for the voluntary wheel running experiments. Control mice (single transgenic or wild-type mice) and PKD1kd–GFP-expressing mice were treated equally with doxycycline-containing drinking water. After 2.5 weeks of voluntary wheel running, all mice were killed and hindlimb crural muscles were dissected for biochemical analysis or cryosectioning and determination of fibre type composition.

Indirect immunofluorescence

Freshly prepared mouse tissue was snap frozen in liquid nitrogen-cooled isopentane. Frozen sections (10–16- μ m thick) were cut on a cryostat (Leica) and transferred to microscope slides (Polysine; Menzel-Gläser) by thaw mounting. Slides were fixed in 4 % paraformaldehyde (Electron Microscopy Science) in PBS for 15 min. For immunofluorescence staining, slides were washed in PBS twice for 5 min each and permeabilized in 0.3 % Triton X-100 in PBS for 5 min. After washing again twice with PBS, the slides were blocked with 5 % goat serum in PBS for 1 h at room temperature (21 °C), followed by incubation with a MHC I-specific antibody (BA-D5) in 5% goat serum at 4°C overnight. Slides were washed three times for 5 min each in PBS and incubated for 1 h at room temperature with Alexa Fluor® 546-coupled goat anti- (mouse IgG) diluted 1:500 in 5 % goat serum in PBS. The slides were washed three times for 5 min each in PBS, fixed once again in 4% paraformaldehyde in PBS at 4°C for 10 min and sequentially treated as described above with a second primary antibody specific for MHC IIa (SC-71) and an NL637-coupled donkey anti-(mouse IgG) as the secondary antibody diluted 1:500 in 5 % goat serum in PBS. Finally, slides were washed twice for 5 min each in PBS and were mounted with Fluoromount-G (SouthernBiotech). To stain F-actin (filamentous actin), slides were incubated with Alexa Fluor® 546-coupled phalloidin (Invitrogen) and washed twice for 5 min each in PBS before mounting. For nuclear counterstaining a 1:2000 dilution of DRAQ5 (Biostatus) or Hoechst 33258 (Sigma-Aldrich) was applied on to the sections directly before mounting and incubated for 10 min at room temperature.

Microscopy, software and statistical analysis

Confocal images were acquired using a confocal laser scanning microscope (TCS SP2; Leica Microsystems) equipped with a 100/1.4 HCX PlanAPO oil-immersion objective. GFP was excited with an argon laser (488-nm line), whereas Alexa Fluor® 546 was excited with a helium–neon laser (543-nm line). DRAQ5 and Cy5 (indodicarbocyanine) were excited with a helium–neon laser at (633-nm line). Wide-field fluorescence and mosaic pictures were recorded using a wide-field microscope (Zeiss Axio Observer.Z1) equipped with the AxioCam MR3 (Carl Zeiss) and a Plan-Apochromat 20 $\times$ 10.8 M27 or an EC Plan-Neofluar 10 $\times$ 10.30 M27 objective (Carl Zeiss). For quantitative image analysis, microscopic pictures were processed further with Adobe Photoshop and the open source software ImageJ using the plugin Cell Counter. The entire plantaris muscle cross section was analysed at $\times$ 20 magnification, with care taken to ensure comparable cross-section locations. An average total number of >600 fibres were counted from each muscle to calculate the means. Quantification of fibre types (type I, Alexa Fluor® 546-stained; type Iia, NL637-stained; type Iib and type IId/x, unstained) was performed by an individual who had no knowledge

of the coding system. Results of voluntary wheel running and fibre typing are presented as means $\pm$ S.E.M. Statistical significance and *P* values were determined by one-way ANOVA and Tukey's multiple comparison test (fibre typing and reporter gene assay) or two-way ANOVA and Bonferroni post-hoc test (running distance).

RESULTS AND DISCUSSION

Generation of a conditional transgenic system for inducible expression of dominant-negative PKD1kd-GFP

There is some evidence that the individual PKD isoforms are functionally redundant in skeletal muscle and heart [16,21], since all three isoforms are expressed in skeletal muscle and cardiac tissue (Figure 1A). To create a functional PKD knockout, we therefore generated transgenic mice expressing a PKD1kd-GFP protein that is known to act dominant-negatively on all three isoforms. The PKD1kd protein harbours a point mutation at position 612, which disrupts kinase activity [27]. The PKD1kd-mediated dominant-negative effect on endogenous PKD signalling has been demonstrated in membrane fission and secretion [22,33], cell migration [34], and phosphorylation of HDAC5 and HDAC7 [13,17]. To express PKD1kd-GFP in an inducible and conditional manner, we made use of the tetracycline-dependent gene expression system originally described by Gossen and Bujard [35]. In this system, the tet-activator protein (rtTA) is expressed constitutively from the activator transgene. In the presence of the tetracycline analogue doxycycline, the rtTA protein binds to the TRE (tetracycline-responsive promoter element), thus inducing the expression of the transgene (Figure 1B). The activator transgene used in the present study was driven by the CMV enhancer/ β -actin promoter, which has been described to induce strong rtTA expression in skeletal muscle tissue, and moderate expression in heart, skin, kidney, thymus and lung [26]. Six independent transgenic mouse lines were generated from founder animals carrying the reporter transgene, which contained the TRE and the gene encoding human PKD1kd-GFP (Figure 1B). Integration of the transgene into the genome was confirmed by Southern blot analysis using a GFP-specific probe (Figure 1C; transgenic line 1 is exemplarily shown). Transgene heredity among offspring was analysed by PCR of the genomic DNA using PKD1-specific oligonucleotides (Figure 1D). One of these lines (line 1) demonstrated high expression of the transgene (Figure 2A) and was selected for further experiments [30]. Double transgenic animals (referred to as CMV/PKD1kd-GFP tg) were created by crossing animals from the activator line to animals from the rtTA-responsive mouse line and were indistinguishable from their single transgenic littermates.

Induction of PKD1kd-GFP expression is dependent on doxycycline and occurs predominantly in skeletal muscle tissue

Administration of doxycycline rapidly induces transgene expression, with induction being complete within 24 h in most organs [36]. First, to analyse whether expression of the PKD1kd-GFP transgene was inducible, double transgenic animals were treated with doxycycline for 1 week. Western blot analysis of double transgenic animals demonstrated high levels of transgene expression in skeletal muscle and, additionally, low expression levels in heart, thymus, skin and stomach (Figure 2A). This is in line with the reported expression pattern of rtTA in the respective transactivator line [26]. Of note, we could not

detect any expression in the brain (results not shown). This is in accordance with findings showing that the originally described rtTA requires relatively high concentrations of doxycycline for full activation. Because of the limited penetration of the blood-brain barrier by doxycycline, this concentration is only reached by supplying doxycycline in the food [37].

To demonstrate that the transgene expression was doxycycline-dependent, double transgenic animals were treated with doxycycline for different times and PKD1kd-GFP expression was analysed in lysates of skeletal muscle tissue by Western blotting (Figure 2B). Transgene expression was already detectable within 3 h after doxycycline administration and increased over time reaching a maximum within 1–3 weeks of treatment. Moreover, PKD1kd-GFP was not detectable in control mice, indicating that expression was strictly doxycycline-dependent and tightly controlled. A prerequisite for the dominant-negative action of PKD1kd-GFP is its considerable overexpression compared with endogenous PKD. We therefore analysed the expression of endogenous and transgenic PKD1 in skeletal muscle tissue of CMV/PKD1kd-GFP transgenic and control mice. Quantitative Western blotting using a PKD1-specific antibody revealed a 13-fold higher expression level of PKD1kd-GFP compared with endogenous PKD1 (Figure 2C), which is the most abundantly expressed PKD isoform in skeletal muscle [21]. Furthermore, we investigated PKD1kd-GFP expression in skeletal muscle tissue at a single-cell level. Fluorescence microscopy of cryosections revealed uniform expression of PKD1kd-GFP in soleus and plantaris muscle (Figure 2D). Confocal laser scanning microscopy revealed that, in addition to a cytoplasmic distribution, catalytically inactive PKD1 was strongly enriched in the nuclei of skeletal muscle cells (Figure 2E, filled arrowheads). In contrast, dominant-negative PKD1kd-GFP was not detected in cells and nuclei of the connective tissue or endothelium within the skeletal muscle (Figure 2E, open arrowheads). Interestingly, active PKD phosphorylates class IIa HDAC proteins in the nucleus, thereby mediating nuclear export and MEF2 activation [14]. Localization of dominant-negative PKD1kd to this compartment should thus allow for the inhibition of active endogenous PKD in the nucleus. Of note, although PKD1kd-GFP was expressed at high levels after 3 weeks of doxycycline treatment (Figure 2B), mice did not demonstrate apparent phenotypic changes. Body weight, heart and hindlimb crural skeletal muscle sizes, as well as fibre type, content of CMV/PKD1kd-GFP transgenic mice were not altered compared with control animals (results not shown). Conversely, animals expressing PKD1ca protein in skeletal muscle tissue demonstrated a lean phenotype accompanied by reduced body weight. This phenotype does not result from an altered metabolism, but rather from an increase in type I fibres and a reduction in myofibre size [21]. Taking into account that skeletal muscle remodelling is induced in response to environmental demands, such as exercise and electrical stimulation [1], one can assume that the activation of the PKD signalling pathways requires such external stimulation. Therefore expression of PKD1kd-GFP will only result in a dominant-negative phenotype under conditions of skeletal muscle remodelling, whereas expression of PKD1ca bypasses external signalling. Indeed, PKD1ca activates MEF2 in skeletal muscle *in vivo* [21]. Furthermore, PKD1ca activates MEF2 transcriptional activity in C2C12 myogenic cells independently of stimulation with acetylcholine, which is reported to stimulate muscle activity in a MEF2-dependent manner in monolayer cell culture [38]. Expression of PKD1wt

increased MEF2 activity, whereas PKD1kd maintained activity of the MEF2 promoter at the basal level in unstimulated and stimulated cells (Supplementary Figure S1 at <http://www.BiochemJ.org/bj/440/bj4400327add.htm>). Whether long-term expression of PKD1kd–GFP might have an effect on skeletal muscle tissues independently of external signals has to be investigated further.

Expression of PKD1kd–GFP decreases running performance and inhibits skeletal muscle remodelling

Wu and co-workers [39] have demonstrated that the transcriptional activity of MEF2 in mice was enhanced by voluntary wheel running and was dependent on the activity of the phosphatase calcineurin. The response was accompanied by an increase in myoglobin expression, resulting in the transformation of type IIb myofibres into type IIa myofibres in plantaris muscle of running mice. Furthermore, calcineurin enhanced the activity of PKD1 to activate slow-twitch- and oxidative-myofibre-specific gene expression thus acting synergistically with PKD1 [21]. It can therefore be speculated that the functional knockout of endogenous PKD may interfere with exercise-induced skeletal muscle remodelling. To address this, mice were given access to a running wheel. As described previously [39], the mice exercised almost continuously mainly during the nocturnal phase of their day/night cycle. This activity was monitored and quantified by counting wheel revolutions. Running performance of control mice (single transgenic supplied with doxycycline) without transgene expression increased steadily within 17.5 days, reaching an absolute running distance of 191.5 ± 15.0 km (Figure 3B) and an average daily running distance of 15.0 ± 2.1 km (Figure 3C). This is in line with previous observations demonstrating an average daily running distance of 10.2 ± 0.7 km [40]. Western blot analysis of skeletal muscle tissue revealed exercise-induced activation of endogenous PKD, evident from enhanced phosphorylation at Ser⁹¹⁶ (Figure 3A). These results are consistent with findings demonstrating enhanced PKD Ser⁹¹⁶ phosphorylation upon treadmill exercise in skeletal muscle [41]. The importance of this observation is supported further by a study showing that Ser⁹¹⁶ phosphorylation plays a critical role in linking electrical stimulation and PKD signalling to myofilament Ca²⁺ sensitivity [42]. However, recently published findings have demonstrated an increase in AMPK α and CaMKII, but not PKD, activity upon exercise [43]. Of note, the authors analysed kinase activity after an acute bout of exercise (60 min of cycling), whereas we observed increased PKD activity after 3 weeks of voluntary wheel running.

Interestingly, mice expressing PKD1kd–GFP demonstrated a significantly decreased ($P < 0.001$) running performance with an absolute running distance of 74.8 ± 22.3 km (Figure 3B) and an average daily running distance of 6.3 ± 1.7 km (Figure 3C). This indicates that expression of the dominant-negative PKD1kd–GFP interferes with the ability of muscles to power exercise-induced contractions. In sedentary, cage-bound mice, MEF2 activity is only detectable in soleus muscle, which is used to support the skeleton against gravity and predominantly contains slow-twitch type I fibres [39]. Wheel-running-induced muscle contractions require the power of soleus, plantaris and white vastus muscles [39]. In soleus muscle, however, the fibre type did not change in response to 60 days of wheel running [44]. In contrast, in plantaris muscle, exercise induced fibre type transformation from type IIb +

IId/x into type Iia, whereas the amount of type I fibres was low and remained unchanged [40]. Therefore we analysed the fibre type composition in the complete plantaris muscle after 18 days of voluntary wheel running using indirect immunofluorescence staining of MHC type I and type Iia (Figure 3D). In control animals, an exercise-induced fibre type transformation from type IId + type IId/x ($82.57 \pm 1.39\%$ compared with $50.22 \pm 0.26\%$; $P < 0.001$) to Iia ($17.23 \pm 1.43\%$ compared with $49.61 \pm 0.127\%$; $P < 0.001$) was clearly visible (Figure 3E). Interestingly, when compared with control animals without the transgene, PKD1kd–GFP-expressing mice contained a significantly higher amount of type IId + type IId/x fibres ($68.58 \pm 1.88\%$ compared with $50.22 \pm 0.26\%$; $P < 0.01$), whereas the amount of type Iia fibres was decreased ($31.37 \pm 1.91\%$ compared with $49.61 \pm 0.13\%$; $P < 0.01$) (Figure 3E). The percentage of type I fibres in plantaris muscle was low and remained unchanged ($0.53 \pm 0.5\%$ in PKD1kd–GFP mice compared with $0.16 \pm 0.1\%$ in control mice) (Figure 3E). In sedentary mice, expression of PKD1kd–GFP did not change fibre type composition compared with sedentary control mice (type Iia fibres, $24.13 \pm 4.39\%$ compared with $17.23 \pm 1.43\%$, $P > 0.05$; type IId + IId/x fibres, $74.90 \pm 5.01\%$ compared with $82.57 \pm 1.39\%$, $P > 0.05$), proving that the dominant-negative action of PKD1kd protein requires a stimulus. In line with this, voluntary wheel running induced a conversion of the fibre type composition from type IId + IId/x into type Iia in control, but not in PKD 1kd–GFP, mice (type Iia fibres, $49.61 \pm 0.13\%$ compared with $31.37 \pm 1.9\%$, $P < 0.01$; type IId + IId/x fibres, $50.22 \pm 0.26\%$ compared with $68.58 \pm 1.88\%$, $P < 0.01$). This indicates that the expression of PKD1kd–GFP is sufficient to block exercise-induced transformation of type IId + IId/x fibres into type Iia fibres in plantaris muscle, thus decreasing running performance. This is supported by a recent study demonstrating that expression of PKD1kd antagonizes the effects of α -adrenergic signalling on the nucleocytoplasmic shuttling of HDAC5 in cultured soleus muscle fibres [7]. Taken together, our results indicate that PKD controls exercise-induced skeletal muscle remodelling in a MEF2-dependent manner. However, we cannot rule out that the expression of PKD1kd–GFP could affect other signalling pathways involved in skeletal muscle remodelling as well. For example, PKD3 has been shown to regulate basal and insulin-induced glucose uptake in L6 myotubes [45]. However, whether PKD is involved in exercise-induced glucose uptake is presently unclear. What is the mode of action of PKD1kd–GFP? It is most likely that the PKD1kd protein competes with the endogenous PKD isoforms for substrate binding. In addition, PKD1kd–GFP could compete with other class Iia HDAC kinases, such as AMPK, Mark2, CaMK and Sik1. Sik1 was reported to promote survival of skeletal myocytes via class Iia HDAC phosphorylation [13], but it is not known whether Sik1 signalling is involved in physiological exercise-induced skeletal muscle remodelling. Likewise, Mark2 is not activated by muscle contraction [46]. Several studies have addressed the role of CaMK in skeletal muscle. Using transgenic mice overexpressing a constitutively active CaMKIV, a role for the kinase in exercise-induced mitochondrial biogenesis and oxidative metabolism has been demonstrated [47]. Although it has been shown that CaMKI and CaMKIV directly phosphorylate and regulate HDAC5 [48], these kinases are not expressed in skeletal muscle [49], making a role in fibre type remodelling unlikely. The major CaMK expressed in skeletal muscle is CaMKII [49], which is activated upon exercise [32]. Studies have demonstrated that CaMKII directly phosphorylates and interacts with HDAC4 via a unique binding motif [10]. Interestingly, although HDAC5 is not a direct target, it gains CaMKII

responsiveness by formation of hetero-oligomers with HDAC4 [9]. Of note, basal and maximal CaMKII activity was unchanged in skeletal muscle from mice expressing PKD1kd–GFP compared with control animals, demonstrating that the dominant-negative PKD1kd protein does not interfere with CaMKII activity (Figure 3F). A recent study demonstrated that, following exercise, nuclear export of HDAC4 and HDAC5 was associated with the activation of AMPK and CaMKII in skeletal muscle, suggesting redundancy of these kinases in signalling to class IIa HDACs [43]. These findings and those of the present study suggest that different kinases, including CaMKII, AMPK and PKD, might be equally important in mediating skeletal muscle remodelling upon exercise.

In conclusion, our *in vivo* model of muscle-specific inducible interference with PKD activity clearly demonstrates the important physiological role of PKD as a key regulator of skeletal muscle fibre type composition and muscle function in general.

Supplementary Material

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Abbreviations used

AMPK	AMP-activated protein kinase
CaMK	Ca ²⁺ /calmodulin-dependent protein kinase
CMV	cytomegalovirus
F-actin	filamentous actin
GFP	green fluorescent protein
EGFP	enhanced GFP
HDAC	histone deacetylase
MEF2	myocyte enhancer factor 2
MHC	myosin heavy chain
PKD	protein kinase D
PKDlca	constitutively active PKD1
PKD1kd	kinase-dead PKD1

PKD1wt wild-type PKD1
TRE tetracycline-responsive promoter element

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Figure 1. Generation of transgenic tetO–PKD1kd–GFP mice

(A) Western blots illustrating endogenous expression of PKD1, PKD2 and PKD3 in skeletal muscle (sm) and heart (he) of wild-type mice. Molecular masses (in kDa) are indicated on the left. IB, immunoblotting. (B) Outline of the rtTA-dependent unit and tetR–VP16 fusion protein. rtTA requires doxycycline for binding to tetO in order to activate transcription. For Tg(tetO–PKD1kd–GFP), the construct used for the generation of transgenic animals is depicted. Restriction enzyme digestion sites that are critical for cloning and for Southern blot analysis are shown as well as the binding site of the 577-bp GFP probe (handle) and the primer binding sites for PCR genotyping (for/rev, arrows). (C) Southern blot analysis of founder genomic DNA demonstrating the presence of the transgenic construct tetO–PKD1kd–GFP integrated as a head-to-tail array. After EcoRV digestion and hybridization with a GFP-specific probe, two fragments were detected: a fragment of predictable size (4.6 kb) due to tandem integration and a junction fragment (>10 kb) with an individual size depending on EcoRV sites flanking the position of transgenic integration, wt, wild-type; tg, transgenic. (D) PCR genotyping demonstrating the presence of the transgenic construct Tg(tetO–PKD1kd–GFP) among offsprings. Amplification of a specific 246-bp fragment indicates transgenity. As an internal control a 399-bp fragment of the endogenous PKD1 sequence was amplified.

Figure 2. Doxycycline-dependent expression of PKD1kd-GFP in double transgenic mice carrying tetO-PKD1kd-GFP and CMV/ β -actin rtTA

(A) Tissue specificity of transgene expression. The densitometry of the skeletal muscle sample was arbitrarily set to 1.0. sm, skeletal muscle; he, heart; st, stomach; ut, uterus; sk, skin; th, thymus; lu, lung; li, liver; ki, kidney; sp, spleen. (B) Kinetics of induction upon doxycycline treatment in skeletal muscle. The densitometry of the 1-week induction sample was arbitrarily set to 1.0. (C) Overexpression level of transgenic PKD1kd-GFP compared with endogenous PKD1 in skeletal muscle. Skeletal muscle from doxycycline (DOX)-treated single transgenic animals carrying tetO-PKD1kd-GFP served as a control. The densitometry of endogenous PKD1 in the control sample was arbitrarily set to 1.0. Representative Western blots and relative (rel.) expression are shown in (A–C). Molecular masses (in kDa) are indicated on the left. IB, immunoblotting. (D) PKD1kd-GFP expression

pattern in hindlimb crural muscle. Bright-field and wide-field fluorescence images are shown. Nuclei were stained with DRAQ5. SO, soleus; PL, plantaris. Scale bar, 100 μm . (E) Subcellular localization of PKD1kd-GFP (green) in skeletal muscle fibres. Nuclei (blue) were stained with DRAQ5 or Hoechst 33258 and are indicated with filled (muscle cell nucleus) or open (non-muscle cell nucleus) arrowheads. F-actin (red) was stained with Alexa Fluor[®] 546-coupled phalloidin. Projections of confocal sections are shown. Scale bar, 10 μm .

Figure 3. Mice expressing PKD1kd-GFP show decreased voluntary wheel running performance and impaired muscle remodelling

(A) Western blot of skeletal muscle lysates isolated from two mice after 3-weeks voluntary wheel running (run) and two sedentary mice (sed) showing an exercise-induced increase in autophosphorylated PKD (Ser⁹¹⁶) levels. Molecular masses (in kDa) are indicated on the left. IB, immunoblotting. (B) Absolute running distance and (C) daily running distance of mice expressing PKD1kd-GFP (O) and control mice without transgene expression (●). Values are mean ± S.E.M. (*n* = 9 for each group); *P* < 0.001. (D) Indirect immunofluorescence staining of MHC type I (red) and type IIa (blue) on plantaris muscle sections from mice expressing PKD1kd-GFP and control mice without transgene expression after 17.5 days of voluntary wheel running. Unstained fibres are type IIb and type IIc/d/x. Scale bars, 100μm (E) Quantitative fibre type analysis of plantaris muscle from mice without (wt) or with (tg) transgenic PKD1kd -GFP expression after 17.5 days of voluntary wheel running or from sedentary control mice. Values are means ± S.E.M. (*n* = 3 for each group). ***P* < 0.01 and ****P* < 0.001. (F) CaMKII was immunoprecipitated (IP) from muscle lysates and kinase activity was determined in the presence (maximal) or absence (basal) of CaCl₂ as the incorporation of γ-³²P into the substrate autocalmitide-2. Quantification was performed by densitometric analysis of the autoradiograms. Western blot analysis shows equal amounts of immunoprecipitated CaMKII and expression of PKD1kd-GFP in skeletal

muscle of doxycycline-treated mice. The histograms represent averaged data (means $\pm$ S.E.M., $n = 2$). Dox, doxycycline; IB, immunoblotting; TCL, total cell lysate.