J Membrane Biol (2011) 242:1–10
DOI 10.1007/s00232-011-9365-7

ORIGINAL ARTICLES

Na,K-ATPase Activity in Mouse Muscle is Regulated by AMPK and PGC-1α

Maria S. Ingwersen · Michael Kristensen · Henriette Pilegaard · Jørgen F. P. Wojtaszewski · Erik A. Richter · Carsten Juel

Received: 23 November 2010 / Accepted: 9 April 2011 / Published online: 18 June 2011

Abstract Na,K-ATPase activity, which is crucial for skeletal muscle function, undergoes acute and long-term regulation in response to muscle activity. The aim of the present study was to test the hypothesis that AMP kinase (AMPK) and the transcriptional coactivator PGC-1α are underlying factors in long-term regulation of Na,K-ATPase isoform (α,β and PLM) abundance and Na^+ affinity. Repeated treatment of mice with the AMPK activator AICAR decreased total PLM protein content but increased PLM phosphorylation, whereas the number of α- and β-subunits remained unchanged. The K_m for Na^+ stimulation of Na,K-ATPase was reduced (higher affinity) after AICAR treatment. PLM abundance was increased in AMPK kinase-dead mice compared with control mice, but PLM phosphorylation and Na,K-ATPase Na^+ affinity remained unchanged. Na,K-ATPase activity and subunit distribution were also measured in mice with different degrees of PGC-1α expression. Protein abundances of α1 and α2 were reduced in PGC-1α +/− and −/− mice, and the β_1/β_2 ratio was increased with PGC-1α overexpression (TG mice). PLM protein abundance was decreased in TG mice, but phosphorylation status was unchanged. Na,K-ATPase V_{max} was decreased in PCG-1α TG and KO mice. Experimentally in vitro induced phosphorylation of PLM increased Na,K-ATPase Na^+ affinity, confirming that PLM phosphorylation is important for Na,K-ATPase function. In conclusion, both AMPK and PGC-1α regulate PLM abundance, AMPK regulates PLM phosphorylation and PGC-1α expression influences Na,K-ATPase α_1 and α_2 content and β_1/β_2 isoform ratio. Phosphorylation of the Na,K-ATPase subunit PLM is an important regulatory mechanism.

Keywords Na,K-pump regulation · PLM phosphorylation · Na,K-pump isoform expression

M. S. Ingwersen · M. Kristensen · H. Pilegaard · C. Juel (✉)
Department of Biology, University of Copenhagen,
August Krogh Building, Universitetsparken 13,
Copenhagen 2100, Denmark
e-mail: cjuel@bio.ku.dk

J. F. P. Wojtaszewski · E. A. Richter
Institute of Exercise and Sport Sciences,
University of Copenhagen, Copenhagen, Denmark

Introduction

Muscle activity induces ion fluxes and changes in ion distribution, which may affect muscle excitability and may lead to impaired force development. Na,K-ATPase (Na,K pump) maintains normal transmembrane gradients for Na^+ and K^+, which is crucial for muscle function. Na,K-pump activity counteracts changes in ion distribution during muscle activity and restores normal ion gradients afterward. Therefore, regulation of the pump is important for muscle function; the complex regulatory mechanisms include hormonal and ion-induced modifications as well as changes in association between subunits. Studies in rats have demonstrated that the kinetic properties of the Na,K pump can change during acute muscle activity, and a change in the phosphorylation of subunits has been suggested to be one underlying mechanism (Juel 2009). The preceding steps for these modifications are not fully understood.

The subunit PLM (phospholemman, FXYD1) is substrate for protein kinases and known to be an important regulator of Na,K-ATPase activity (Crambert et al. 2002). The general scheme, obtained from studies in expression systems, is that binding of unphosphorylated PLM to the

$\alpha\beta$ complex of the pump reduces the Na^+ affinity, whereas PKA-induced phosphorylation of PLM increases the affinity for Na^+ (Bibert et al. 2008; Crambert et al. 2002: Despa et al. 2005; Lifshitz et al. 2006). However, the physiological role of this mechanism is not clear.

The intracellular energy sensor 5′-AMP-activated protein kinase (AMPK) is activated by muscle contractions and is thought to play an important role in the acute regulation of metabolism in skeletal muscle (Jensen et al. 2009). In rats, injection of aminoimidazole-4-carboxymide-1-β-D-ribofuranoside (AICAR), which is often used as a tool to stimulate AMPK activity, induced an acute fall in the plasma K^+ concentration after 3 h (Zheng et al. 2008), suggesting that AMPK activation may be a potential mechanism regulating Na,K-pump function during muscle activity. However, AICAR infusion had no effect on maximal ATPase activity measured in muscle homogenates, and the plasma membrane abundance of the α_2-subunit was unchanged (Zheng et al. 2008). It has been suggested that AMPK activation could be involved in Na,K-ATPase subunit translocation to the outer membrane, leading to a higher density of active subunits (Benziane and Chibalin 2008). However, in one study a 20% decrease in plasma membrane abundance of the α_2-subunit after acute AICAR treatment was found (Kristensen et al. 2008). Based on these contradictory results, it can be concluded that the acute mechanism underlying the AICAR-induced changes in plasma K^+ remains unresolved.

It is well known that Na,K-ATPase undergoes changes with training; a number of studies in rats and humans have revealed increased Na,K-ATPase protein content after both sprint and endurance training (Juel 2006). Since AMPK may have a role in the molecular adaptations to exercise (Jensen et al. 2009; Winder et al. 2006), it is a possibility that long-term AMPK activation/deactivation may be one underlying factor regulating Na,K-ATPase. The present study investigates the long-term effects of AMPK activation and inhibition on PLM abundance and Na,K-ATPase activity. For that purpose, we used repeated AICAR infusion to stimulate AMPK in wild-type (WT) mice. Other experiments used "kinase-dead" (KD) mice overexpressing a dominant negative KD α_2-AMPK construct to quantify the effects of reduced AMPK activity.

One of the targets of AMPK is the transcriptional coactivator peroxisome proliferator–activated receptor-γ coactivator-1α (PGC-1α). PGC-1α transcription and mRNA are increased in rodent and human skeletal muscle in response to a single exercise bout, and although not mandatory (Leick et al. 2009), PGC-1α is most likely an important factor in exercise training–induced adaptations in skeletal muscle oxidative capacity (Olesen et al. 2010). Increased PGC-1α expression stimulates mitochondrial biogenesis and mediates phenotypic changes toward the appearance of the more oxidative metabolism associated with slow-twitch fibers (Lin et al. 2002).

The expression of PGC-1α is depressed in type II diabetic patients and with increasing age (reviewed by Olesen et al. 2010), and training led to an increased level of PGC-1 mRNA (Pilegaard et al. 2003) and an increased PGC-1 protein content (Burgomaster et al. 2008). In rat epitrochlearis muscle PGC-1 mRNA is elevated after swimming exercise training (Gato et al. 2000; Terada et al. 2002). Because Na,K-ATPase follows the same expression pattern (Dela et al. 2004), it is likely that PGC-1α contributes to the activity-induced changes in Na,K-ATPase. It is therefore hypothesized that PGC-1α mediates some of the changes in Na,K-ATPase activity seen with muscle activity/inactivity and that changes in PGC-1α expression influences Na,K-ATPase properties. Because the Na,K-ATPase isoform distribution, enzyme activity and Na^+ affinity are strongly dependent on fiber types (Kristensen and Juel 2010); and because the PGC-1α level is expected to influences fiber type (Lin et al. 2002), PGC-1α could mediate changes of expression of subunits and concomitantly influence Na,K-ATPase function. We therefore investigated the possible effects of changes in PGC-1α expression on Na,K-ATPase activity and isoform expression in the untrained state. For that purpose we compared Na,K-ATPase activity and subunit distribution in PGC-1α WT mice, mice overexpressing PGC-1α (TG mice), PGC-1α heterozygotic mice (+/− mice) and PGC-1α knockout (KO) mice.

Methods

Animals and Ethical Approval

The handling of animals was conducted in accordance with Danish animal welfare regulations. Mice were provided with unlimited food and water and kept under a 12/12 h dark/light cycle. The breeding and genotyping of the animals have been described elsewhere (Leick et al. 2008).

The effect of AMPK depression was studied in KD mice overexpressing a KD α_2-AMPK construct, which inhibits expression of other AMPK isoforms (Jensen et al. 2007).

Na,K-ATPase isoform distribution and enzyme activity were also studied in male C57BL/6 PGC-1α WT mice, mice overexpressing PGC-1α (TG mice), heterozygote +/− mice and KO mice. The generation of these mice has been described elsewhere (Lin et al. 2002, 2004). In TG mice the PGC-1α level is obtained with a muscle creatine kinase (MCK) promoter, which induces a fiber-type conversion (Lin et al. 2002). Each strain had its own WT

(littermates to PG and KO, respectively), and both contributed to the WT group in the present study.

Repeated AICAR Treatment Protocol

WT female mice (C57BL/6, 4–6 months of age) were given daily subcutaneous injections of AICAR (500 mg/kg body weight; Toronto Research Chemicals, North York, Canada) dissolved in 0.9% NaCl or injections of saline. AICAR was given over a 27-day period, with injections every second day the first 6 days and every day during the rest of the period. Mice were anesthetized 24 h after the last AICAR injection by an intraperitonal injection of pentobarbital sodium (6 mg/100 g body weight). Quadriceps femoris muscles were used for Na,K-ATPase measurements. Other aspects (changes in AMPK phosphorylation, VEGF and HKII protein expression) of the experiments have been described elsewhere (Leick et al. 2009).

Muscle Preparations and Membrane Fractionating: Total Membranes

All sample preparations were carried out at a maximum temperature of 4°C unless otherwise stated. The protein content of samples was determined using a bovine serum albumin (BSA) standard (DC protein assay; Bio-Rad, Richmond, CA). To characterize isoform distribution in different muscles, we dissected soleus and red gastrocnemius (representing red muscle), extensor digitorum longus, white gastrocnemius and white vastus lateralis (representing white muscle). It must be noted that "red" mouse muscle has mixed fiber types; consequently, it is not possible to dissect only red muscle fibers. If not otherwise stated, the following experiments were carried out with mixed hind-limb muscle.

After mincing, mouse hind-limb muscle samples were homogenized for 30 s (Polytron PT 2100; Kinematica, Lucerne, Switzerland) in 250 mM mannitol, 30 mM L-histidine, 5 mM EGTA and 0.1% deoxycholate (pH 6.8). The sample (crude homogenate) was subjected to 3,000×*g* spinning for 30 min, and the supernatant was subjected to 190,000×*g* spinning for 90 min (4°C). The final pellet, called "total membranes," was resuspended in assay buffer (see below) and used for activity measurements. Sample protein recovery was about 5% of total protein content (including soluble proteins) in the crude homogenate, and the Na,K-ATPase α_2 isoform protein content in the final total membrane fraction (quantified with Western blotting) was more than half of the content in the crude homogenates. The production of total membranes removed some of the background ATPase activity, thereby increasing the maximal Na^+-stimulated Na,K-ATPase activity to 10–20% of the total ATPase activity (including Ca^{2+} ATPase).

Measurement of Na^+-Stimulated Na^+,K^+-Atpase Activity in Total Membranes

Na^+-stimulated Na^+,K^+-ATPase activity was determined by measuring the hydrolysis of ATP. Released P_i was detected with the malachite-based Biomol green reagent (Biomol AK-111; Enzo Life Sciences, Exeter, UK) as previously described (Juel 2009; Kristensen and Juel 2010). Total membrane (200 μg of protein) was suspended in assay buffer (10 mM KCl or 80 mM NaCl, 5 mM $MgCl_2$, 50 mM Tris-base, 5 mM EGTA, pH 7.4). Each sample contained 10 μg of protein. Na^+ was added to the samples to a final concentration of 0, 2, 4, 6, 10, 20, 40 or 80 mM (ionic strength was kept constant by substituting NaCl with choline chloride). After 15 min of preincubation at 37°C, the reaction was started by adding Mg-ATP (Sigma, St. Louis, MO) to a final concentration of 0.5 mM. After 30 min, the samples were placed on ice and the reaction was terminated by adding 1 ml Biomol green reagent. After 30 min, absorbance was read at 620 nm and P_i was calculated from a standard curve. All samples were run in duplicate (0 mM Na^+ four times), and ATPase activity at 0 mM Na^+ was subtracted from all activity values. Preliminary experiments demonstrated that Na^+-stimulated activity was completely inhibited by preincubation with 2 mM ouabain (data not shown).

In Vitro Phosphorylation/Dephosphorylation

Dephosphorylation

Two hundred micrograms of the total membrane fraction was treated with 100 U calf intestinal alkaline phosphatase (CIF, M0290S; New England BioLabs, Beverly, MA) at 37°C for 60 min, diluted to remove background phosphate and sedimented by 20,000×*g* spinning at 4°C; and the pellet was used for the ATPase assay.

Phosphorylation

To simulate PKA-induced phosphorylation, 200 μg of the total membrane fraction was treated with 2 mM cAMP + 2 mM ATP + 20 mM of *β*-glycerophosphate (phosphatase substrate) at 37°C for 90 min, diluted and sedimented to remove background phosphate; and the pellet was used for the ATPase assay.

Western Blotting

Samples were mixed with sample buffer (2 mM Tris–HCl, 0.2 mM EDTA, 20 mM DTT, 4% SDS, 10% glycerol, 0.04% bromophenol blue, pH 8.0). Equal amounts of protein were loaded into each lane and separated by 8–18% SDS-PAGE

(Excel 8–18% gradient gel; GE Healthcare, Uppsala, Sweden) or 12.5% SDS-PAGE (ExcelGel, GE Healthcare) for PLM measurements. The proteins were then electroblotted onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA). The membrane was blocked for 60 min at room temperature in TS buffer (10 mM Tris-base, 0.9% NaCl, pH 7.4) containing 2% BSA, 1% skim-milk powder and 0.1% Tween-20 before incubation with primary antibody diluted in a similar buffer overnight (4°C). After treatment with a horseradish peroxidase–coupled secondary antibody (Dako, Glostrup, Denmark) for 90 min at room temperature, the membrane was repeatedly washed in TS buffer with or without 0.05% Tween-20. The membrane was incubated with enhanced chemiluminescence reagent (ECL, GE Healthcare) and visualized on Hyperfilm (GE Healthcare). Samples to be compared were loaded on the same gel. Relative protein concentrations were quantified by scanning the film and analyzing band intensities with UN-SCAN-IT version 5.1 software (Silk Scientific, Orem, UT). For each Na,K-ATPase isoform the abundance in the experimental animals was presented relative to the mean of the amount in control animals. This method does not allow comparison of different isoform protein levels.

Antibodies

The α_1 isoform of Na/K-ATPase was detected with the monoclonal α6F antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA). A monoclonal antibody (McB2) used to detect the α_2 isoform and a polyclonal antibody to detect the β_1 isoform were generously provided by Dr. P. A. Pedersen (University of Copenhagen, Copenhagen, Denmark). The β_2 isoform was detected with a polyclonal antibody (06-171l; Millipore). For detection of total PLM (FXYD1), the polyclonal C2 antibody (generously provided by Dr. J. Cheung, Geisinger Medical Center, Danville, PA) was used, and the polyclonal CP68 antibody (kindly provided by Dr. D. Bers, Loyola University, Chicago, IL) was used for detection of PLM phosphorylated at serine-68. Caveolin 3 (CAV-3) antibodies (ab2912) were obtained from Abcam (Cambridge, MA).

Statistics and Calculations

Samples to be compared were run on the same gel and with the same amount of protein per lane. Student's unpaired *t*-tests were used to compare relative changes in protein subjected to Western blotting. Apparent affinity constant $K_{0.5}$ (K_m) and V_{max} for Na^+-stimulated ATPase activity were determined for each individual experiment by nonlinear regression (SigmaPlot software; Systat, San Jose, CA) with a Hill equation in the following form: rate of ATP hydrolysis $= (V_{max}\ [Na]^b)/([Na]^b + K_m^b)$, with a variable Hill coefficient (b). A two-way ANOVA for repeated measurements was used to test the effect of time and genotype on Na^+-stimulated Na^+,K^+-ATPase activity. Post hoc analysis was performed using Tukey's test. The mean K_m and V_{max} values were compared using an unpaired *t*-test (control vs. treated samples). A paired test was used to compare phosphorylated/dephosphorylated samples. $P < 0.05$ was considered significant.

Results

Effect of Repeated AICAR Injections on Na,K-ATPase Subunit Abundance

The Western blotting technique was used to quantify changes in the abundance of the Na,K-ATPase subunits after repeated AICAR treatment (Fig. 1a). AICAR injection had no effect on the amount of the membrane marker CAV-3 or on the abundance of the ATPase isoforms α_1, α_2, β_1 and β_2. In contrast, AICAR treatment lowered PLM abundance by 40% ($P < 0.05$), quantified with the C2 antibody, whereas the abundance of phosphorylated PLM measured with the CP68 antibody remained unchanged. These measurements indicate that the degree of PLM phosphorylation was increased in AICAR-treated animals.

Effect of Repeated AICAR Injections on Na,K-ATPase Activity

The Na^+-dependent ATPase activity was measured in control samples and in samples from AICAR-treated animals. AICAR treatment reduced the K_m for Na^+ from 8.0 ± 0.6 to 5.7 ± 0.7 mM ($P < 0.05$). Na,K-ATPase V_{max} remained unchanged in the AICAR-treated animals relative to control (758 ± 61 in control vs. 853 ± 107 nmol ATP mg^{-1} protein h^{-1} in AICAR-treated animals) (Fig. 1b).

Abundance of Pump Subunits in AMPK KD Mice

The abundance of the Na,K-ATPase subunits α_1, α_2, β_1 and β_2 was unchanged in AMPK KD mice compared with WT control mice, whereas the content of both PLM protein and phosphorylated PLM was increased by ~40% in AMPK KD mice compared with WT ($P < 0.05$). The increase in PLM and phosphorylated PLM were not different, indicating that the degree of phosphorylation remained unchanged (Fig. 2a).

Na,K-ATPase Activity in AMPK KD Mice

The Na^+-dependent ATPase activity was measured in WT control samples and in samples from AMPK KD mice. K_m

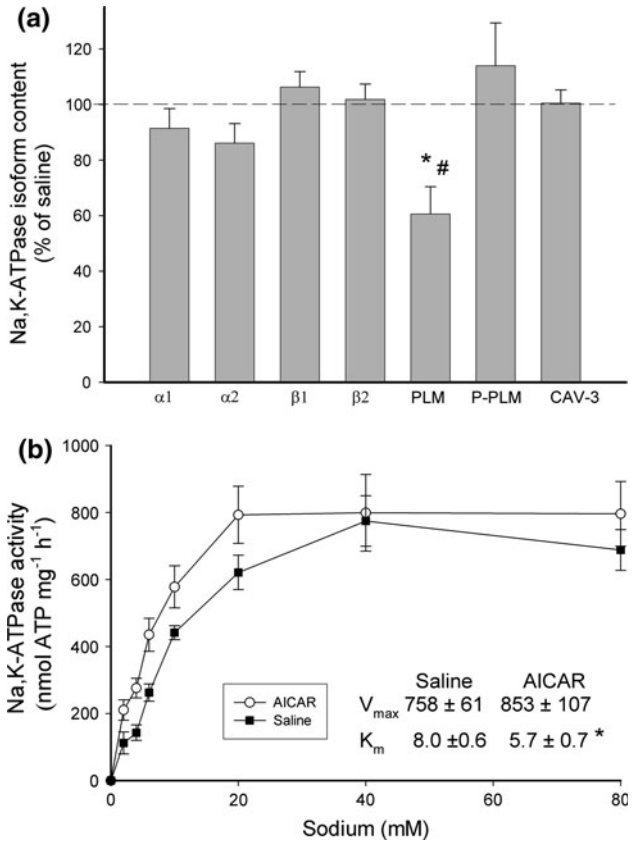

Fig. 1 **a** AICAR injection and Na,K-ATPase isoforms. Western blot analysis was used to quantify the Na,K-ATPase isoform protein abundance in control (saline) mice and in animals treated with AICAR. *Bars* represent the mean value for each protein in AICAR-treated animals calculated relative to the mean of saline-treated animals. The amount of caveolin 3 (*CAV-3*) served as a membrane marker ($n = 8$). *Significantly different from saline, #significantly different from P-PLM. **b** AICAR injection and Na,K-ATPase activity. Na,K-ATPase activity was measured in quadriceps femoris muscle from saline-treated and AICAR treated animals ($n = 8$). K_m and V_{max} were calculated from each experiment, and the mean values ± SE are indicated. *Significantly different from saline

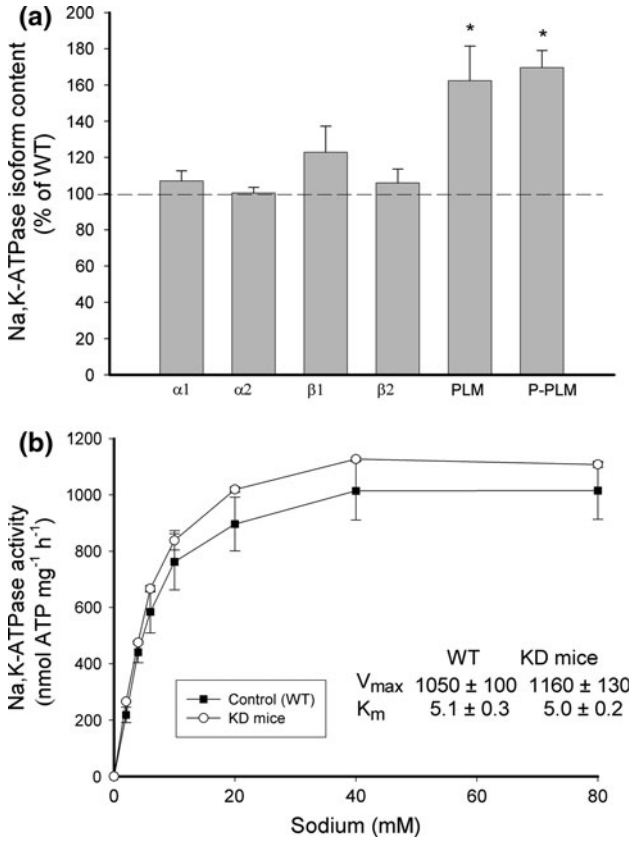

Fig. 2 **a** Na,K-ATPase isoforms in kinase-dead (KD) mice compared with wild-type (WT) mice. Western blot analysis of mixed hind-limb muscle was used to quantify the Na,K-ATPase isoform protein abundance in control WT and KD mice. *Bars* represent mean values in KD animals calculated relative to the mean WT value for each protein ($n = 8$). P-PLM quantified with the CP68 antibody. *Significantly different from WT. **b** Na^+-dependent Na,K-ATPase activity in KD. Na,K-ATPase activity was measured in WT and KD mice ($n = 8$). K_m and V_{max} were calculated from each experiment, and the mean values ± SE are inserted in the figure. None of the parameters was significantly different

for Na^+ in KD mice was not different from WT (5.0 ± 0.2 vs. 5.1 ± 0.3 mM). Also, V_{max} remained unchanged (1,160 ± 130 in KD mice vs. 1,050 ± 100 nmol ATP mg^{-1} protein h^{-1} in WT) (Fig. 2b).

In Vitro Phosphorylation/Dephosphorylation

The degree of phosphorylation after the Western blotting procedure was at least three times higher in the phosphorylated than in the dephosphorylated samples (Fig. 3a).

Figure 3b compares the Na^+-dependent ATPase activity in the in vitro phosphorylated and the dephosphorylated samples. K_m for Na^+ in the dephosphorylated samples (7.5 ± 0.9 mM) was higher than in the phosphorylation samples (4.3 ± 0.7 mM) ($n = 12$, $P < 0.01$). V_{max} was reduced from 548 ± 44 in the dephosphorylated samples to 432 ± 30 nmol ATP mg^{-1} protein h^{-1} in the phosphorylated samples ($P < 0.05$). For the phosphorylated samples the mean activity at 80 mM Na^+ was lower than at 40 mM Na^+, which could influence the K_m and V_{max} values obtained from curve fitting. Without the last data point, K_m and V_{max} were 5.2 ± 1.4 mM and 497 ± 72 nmol ATP mg^{-1} protein h^{-1}, respectively. Calculated in this way, only the mean K_m value for the phosphorylated samples was different from the unphosphorylated samples.

Na,K-ATPase Isoforms and PGC-1α Expression

The distributions of α_1 and α_2 isoforms were not different in red compared to white muscle probed with antibodies.

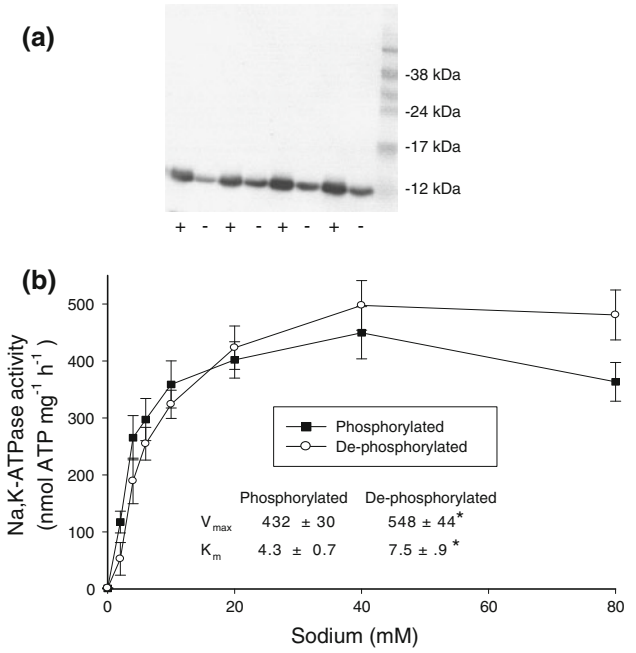

Fig. 3 **a** Representative Western blot of in vitro phosphorylated/dephosphorylated samples probed with the phosphospecific PLM antibody CP68. +, In vitro phosphorylated sample; −, in vitro dephosphorylated sample; to the right, superimposed molecular weight markers. **b** Effects of in vitro phosphorylation/dephosphorylation. Samples were split in half, one phosphorylated and the other dephosphorylated. The ATPase activity at different Na^+ concentrations was measured in each sample, and values are ±SE ($n = 12$). K_m and V_{max} were calculated from each experiment, and the mean values ± SE are inserted in the figure. *Significantly different from phosphorylated samples

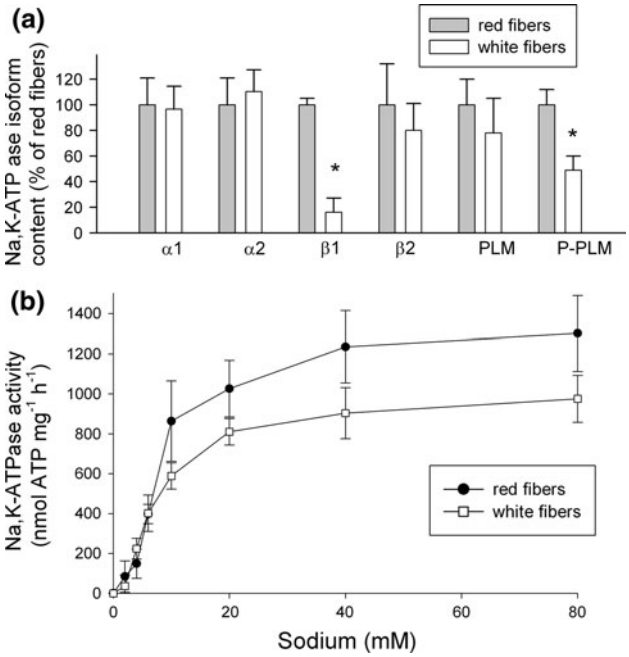

Fig. 4 **a** Distribution of α and β isoforms and phosphorylated and unphosphorylated PLM in red and white muscle obtained by Western blots. For each isoform the relative abundance in white fibers is calculated relative to the mean abundance in red fibers. $n = 6$, ±SE given. *Different from red fibers. **b** Na^+-stimulated Na,K-ATPase activity in red and white muscle fibers. Values are mean ± SE, $n = 6$

The β_1 density was 16% in white muscle (see methods) compared with red muscle ($P < 0.05$), whereas for β_2 the density in white fibers was 80% that in red fibers ($P = 0.1$). Thus, red muscles are characterized by a high density of β_1, whereas β_2 is more uniformly distributed (Fig. 4a). Phosphorylated PLM (P-PLM) was significantly higher in red compared to white fibers, and unphosphorylated PLM tended to be higher in red compared to white fibers. The Na^+-stimulated ATPase activity was 30% higher in red compared to white fibers, whereas the K_m values for Na^+ activation in red and white fibers were not different (7.7 vs. 8.1 mM) (Fig. 4b). It is not possible to dissect pure red and white muscle fibers in mice; the difference in K_m for red and white fibers is therefore underestimated.

Na,K-ATPase isoform distribution was compared in mixed muscle from PGC-1α WT mice, mice overexpressing PGC-1α (TG), +/− mice and −/− mice. The distribution of isoforms was calculated relative to the appearance in WT. α_1 and α_2 protein abundance was reduced in +/− and −/− mice compared with WT without changes in the ratio between isoforms. The β_1 protein expression was high with high PGC-1α expression, whereas β_2 protein expression was low in mice with high PGC-1α expression; thus, high expression of PGC-1α significantly favors the appearance of the β_1 isoform rather than the β_2 isoform. In addition, the total β content was lower in TG compared to WT mice. The total amount of PLM was reduced in mice overexpressing PGC-1α but without changes in the degree of phosphorylation (Fig. 5a).

Na,K-ATPase Activity in PGC-1α-Overexpressed Muscle and KO Muscle

V_{max} was significantly reduced in TG mice to 49% of the level in WT mice (Fig. 5b), whereas K_m remained unchanged (8.3 vs. 8.5 mM). V_{max} was reduced in KO mice to 55% of the level in WT, whereas K_m was unchanged.

Discussion

The main findings of the present study are (1) that AMPK activity influences the PLM density and degree of phosphorylation, (2) that PGC-1α expression influences α and PLM isoform abundance and β isoform distribution and (3) that the degree of PLM posphorylation correlates with Na,K-ATPase Na^+ affinity.

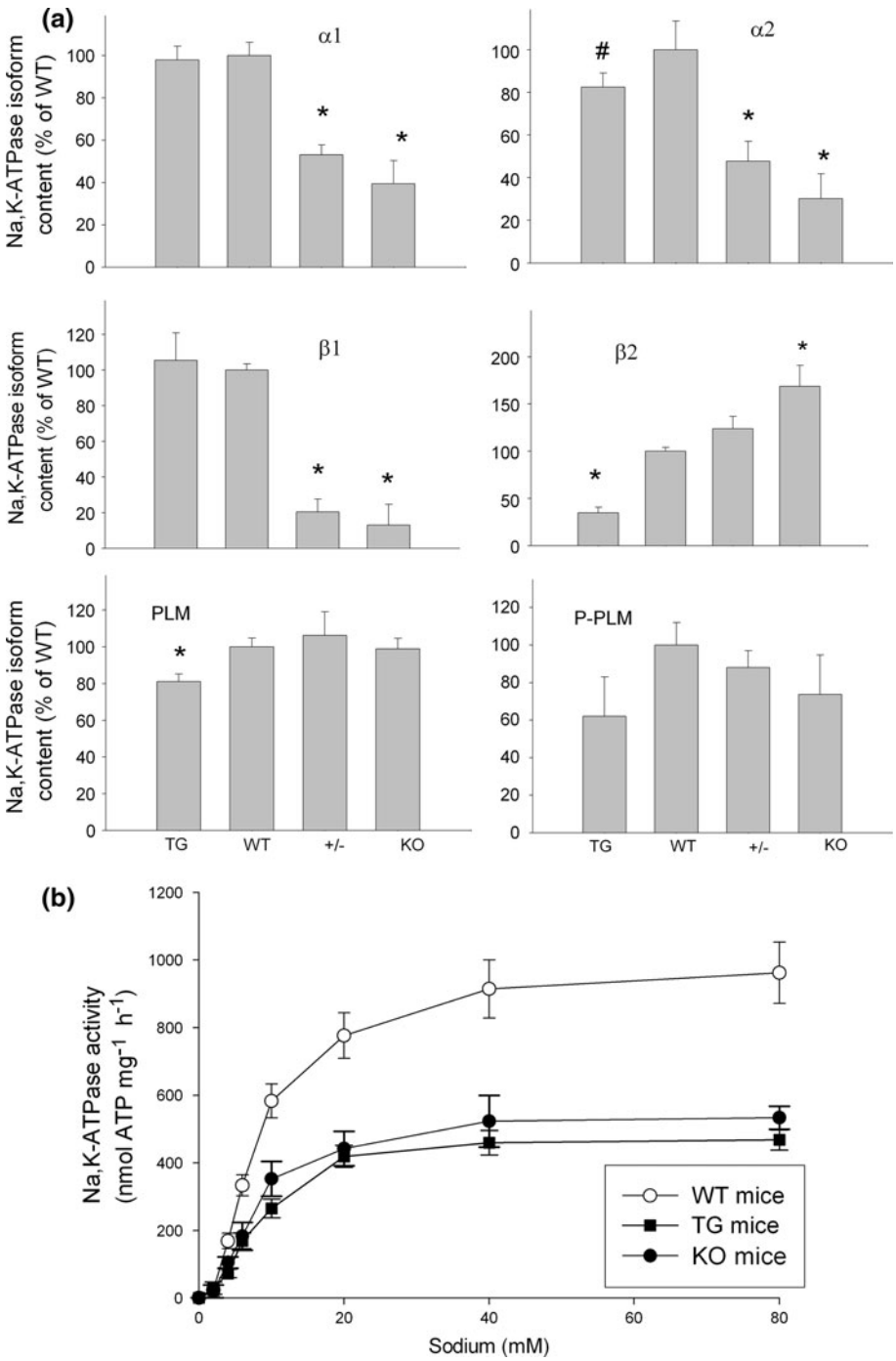

Fig. 5 **a** ATPase subunits and PGC-1α expression. ATPase subunits and PLM phosphorylation were measured in mixed hind-limb muscle from mice with different PGC-1α expression. *TG* mice overexpressing PGC-1α, *WT* heterozygote +/−, *KO* knockout mice. Values are relative to mean WT and given as mean ± SE. $n = 12$ for TG and WT, $n = 6$ for +/− and KO. *Significantly different from WT ($P < 0.05$). $^{\#}P < 0.1$ for TG compared to WT. **b** Na^+-stimulated Na,K-ATPase activity in TG, WT and KO mice. Values are mean ± SE, $n = 12$, 16 and 5, respectively

Role of AMPK

The long-term effect of AMPK activity on Na,K-ATPase function was studied with two models: (1) activation of AMPK with AICAR and (2) the use of AMPK KD mice.

AICAR is often used as a tool to stimulate AMPK activity (Jørgensen et al. 2007), but other AMP-activated pathways, such as p38 MAPK, cannot be excluded. Repeated AICAR injections over a 27-day period were used to mimic daily exercise-induced AMPK activation. Repeated AICAR injections selectively decreased the amount of PLM protein but increased the fraction of P-PLM (ratio between signal with the CP68 and C2 antibodies), without affecting the amount of α- and β-subunits (Fig. 1a). The reduced K_m after AICAR treatment (Fig. 1b) reflects an increased Na^+ affinity, which may be the result of two mechanisms: Firstly, the association between αβ dimers and PLM is known to increase K_m for Na^+ in

expressions systems (Crambert et al. 2002). The reduced K_m could therefore be the result of the reduced content of PLM if PLM binding is dependent on total amount. Secondly, experiments with expression systems and cardiac cells have shown that K_m for Na^+ is reduced with PLM phosphorylation (Bibert et al. 2008; Despa et al. 2005; Lifshitz et al. 2006). The increased degree of PLM phosphorylation after AICAR treatment could therefore also be important for the reduced K_m for Na^+. It is not possible based on the present observations from AICAR treatment to discriminate between effects induced by a decrease in total PLM protein and effects associated with changes in the degree of PLM phosphorylation. Interestingly, AMPK activity is reported to increase PKC-zeta activity (Chen et al. 2002; Vadasz et al. 2008), which could be the link between AMPK and PLM phosphorylation; but other pathways, including PGC-1α, could also be involved (discussed below).

We also used KD mice to study the importance of AMPK activity for ATPase function. KD mice had a higher PLM protein content compared to WT mice, but the degree of phosphorylation was unchanged (Fig. 2a). The K_m for Na^+ in KD mice was not different from that in WT mice (5.0 ± 0.2 vs. 5.1 ± 0.3 mM). These results, together with the AICAR experiments, led to the conclusion that degree of PLM phosphorylation is more important for Na,K-ATPase Na^+ affinity than the total amount of PLM.

In an attempt to illustrate the importance of PLM phosphorylation, in vitro phosphorylation/dephosphorylation of homogenized muscle samples was performed. PKC stimulation was used as an experimental tool because this leads to a specific PLM phosphorylation at Ser68. These experiments clearly demonstrated that the K_m for Na^+ was lower in samples with a high degree of PLM phosphorylation at Ser68 (Fig. 3), which supports the second possibility above that the underlying mechanism in the AICAR study is that PLM phosphorylation affects K_m for Na^+. It must, however, be noted that the in vitro phosphorylation/dephosphorylation used in the present study may also have affected other phosphorylation sites on PLM, e.g., the Ser63 phosphorylation site (Bibert et al. 2008), and that the α-subunit also possesses possible serine phosphorylation sites, which could affect pump function (Feschenko et al. 2000; Zhang and Ng 2007).

A number of studies have evaluated changes in Na,K-ATPase activity from changes in the V_{max} value measured at high (unphysiological) Na^+ concentrations. In the present study V_{max} was unchanged with AICAR and in KD mice and slightly reduced with artificial PLM phosphorylation. However, the physiological importance of a given intervention is better evaluated at physiological Na^+ concentrations. It can be noted that the reduced K_m for Na^+ activation of Na,K-ATPase in the AICAR experiments led to higher activity compared with saline at low Na^+ concentrations (Fig. 1) (see also "Physiological implications", below).

AMPK Studies as a Model for Muscle Activity

AMPK activity is known to be acutely increased with muscle activity and to be involved in a number of metabolic and genetic changes (Jensen et al. 2009; Jørgensen et al. 2007; Leick et al. 2009; Winder and Hardie 1996). The activation/deactivation of AMPK used in the present study and the induced changes in ATPase activity may therefore simulate the changes during repeated muscle activity. The present observation that manipulations in AMPK activity had no effect on Na,K-ATPase α- and β-subunit protein abundance indicates that, although AMPK activity is likely to be involved in activity-induced changes in PLM and PLM phosphorylation, it does not seem to be involved in the general increase in Na,K-ATPase α- and β-subunit protein content seen in most training studies (Juel 2006). Although an increase in α-subunits with training seems to be the general scheme, exceptions have been reported; 5 days of endurance swim training in rats has been shown to increase PLM phosphorylation and membrane Na,K-ATPase activity without changes in total α-subunits quantified with ouabain binding (Galuska et al. 2009). As PLM protein content and degree of phosphorylation have not been measured in most published training studies, the potential role of PLM in the training responses is not yet clarified.

Role of PGC-1α

One downstream effect of AMPK is increased PGC-1α activity. PGC-1α expression is increased with exercise and is thought to be a key regulator of training-induced adaptations in skeletal muscle (Gato et al. 2000; Pilegaard et al. 2003; Terada et al. 2002). A large number of studies have demonstrated increased Na,K-ATPase protein expression with both endurance and sprint training (Juel 2006). It is therefore a possibility that PGC-1α is involved in the changes in Na,K-ATPase activity associated with muscle activity. Here, we investigated a possible correlation between PGC-1α expression and Na,K-ATPase isoform abundance and enzyme activity.

Low expression of PGC-1α in +/− and −/− mice was associated with a low density of both α_1 and α_2 isoforms of Na,K,ATPase without a clear shift in the ratio (Fig. 5a). The β_1/β_2 isoform ratio was clearly correlated with the expression of PGC-1α. A high β_1/β_2 protein ratio, which is associated with oxidative muscle (Juel 2009, and Fig. 4 present study), was found in mice overexpressing PGC-1α, whereas a low ratio was found in PGC-1α +/− and KO

mice. This is in line with the finding that PGC-1α overexpression drives the formation of more oxidative fibers (Lin et al. 2002). Interestingly, 5-day endurance swim training (3-h bouts) in rats has been reported to increase the β_1/β_2 mRNA ratio in gastrocnemius muscle more than fourfold (Galuska et al. 2009). The present finding supports the possibility that PGC-1α is involved in mediating such changes. Overexpression of PGC-1α reduced the absolute amount of the PLM subunit, without changes in the degree of phosphorylation.

The ATPase activities in both TG and KO mice were reduced compared to WT (Fig. 5b). The reduced V_{max} in TG was at first a surprise because oxidative fibers were found to have a high V_{max} compared to glycolytic fibers (Fig. 4b). The reduced V_{max} in TG is likely the result of the dramatic reduction in β_2 (which was not compensated by a similar increase in β_1) and to a minor extent the result of reductions in α_1 and PLM. The reduced V_{max} in mice overexpressing PGC-1α is clearly in contrast to the hypothesis that PGC-1α expression correlates with Na,K-ATPase activity. However, the study shows that PGC-1α expression can modify the important regulatory PLM protein. The reduced V_{max} in KO mice is in agreement with the lower α_1 and α_2 contents compared to WT, whereas the reduced β_1 content to some degree is compensated by an increase in β_2 content.

The disproportional changes in α and β isoforms may be surprising as the functional Na,K-pump is a $\alpha\beta$ dimer; however, it is known from rat muscle that β can be present in surplus. Whether this is also the case in mice is unknown.

It must be noted that the AMPK and PGC-1α experiments cannot be clearly separated: One effect of AMPK is to induce PGC-1α expression and activity (Terada et al. 2002). However, the effect of repeated AICAR injection does not seem to be exclusively through PGC-1α activity because PGC-1α overexpression resulted in a dramatic change in β-subunit ratios, whereas AICAR treatment had no effect on this subunit ratio. In addition, AMPK activation leads to PLM phosphorylation, which was not seen in mice overexpressing PGC-1α.

PGC-1α overexpression has been reported to improve fatigue resistance in mice (Lin et al. 2002). Theoretically, this could be due to improved ion handling in mice overexpressing PGC-1α; however, such a mechanism is not supported by the changes in ATPase isoform distribution and activity reported here. More likely the high fatigue resistance with PGC-1α overexpression is dependent on metabolic changes as originally suggested.

Physiological Implications

It can be questioned if the changes in K_m obtained with homogenized muscle as a model system are of physiological importance because the K_m values with AICAR treatment (8.0 → 5.7 mM) and with artificial phosphorylation (7.5 → 4.3 mM) are low compared to the intracellular Na^+ concentration in intact muscle (~15 mM), at first indicating a high degree of saturation with Na^+. However, the ATPase proteins in the present study were not exposed to the gradients for Na^+ and K^+ ions present in intact cells, which may have affected K_m. The K_m for Na^+ in intact cell is difficult to obtain; one study suggests a K_m of 25–30 mM in rat soleus muscle (Buchanan et al. 2002), which implies only partial saturation. With a similar level for K_m in mice and with the expectation that the direction and relative magnitude of K_m changes in the present study are true, the reported PLM associated K_m changes have pronounced effects on Na,K-ATPase rate in intact cells, which could affect K^+ uptake by active muscle.

In conclusion, the present study revealed some of the pathways involved in regulation of Na,K-ATPase protein abundance and function. Phosphorylation of the PLM subunit seems to be an especially important mechanism. The underlying pathways may be important both for acute and long-term regulation of Na,K-ATPase expression and thereby for regulation of muscle ion homeostasis.

Acknowledgments The present study was supported by the Carlsberg Foundation. We thank Prof. B. Spiegelman for providing PGC-1α mice and M. J. Birnbaum for providing KD mice initially to start breeding in house. We also thank Joachim Fentz, Lotte Leick and Jonas T. Treebak for help handling animals and Helle Walas for skilled technical assistance.

References

Benziane B, Chibalin AV (2008) Frontiers: skeletal muscle sodium pump regulation: a translocation paradigm. Am J Physiol Endocrinol Metab 295:E553–E558

Bibert S, Roy S, Schaer D, Horisberger JD, Geering K (2008) Phosphorylation of phospholemman (FXYD1) by protein kinase A and C modulate distinct Na,K-ATPase isoenzymes. J Biol Chem 283:476–485

Buchanan R, Nielsen OB, Clausen T (2002) Excitation- and β_2-agonist-induced activation of the Na^+-K^+ pump in rat soleus muscle. J Physiol 545:229–240

Burgomaster KA, Howarth KR, Phillips SM, Rakobowchuk M, Macdonald MJ, McGee SL, Gibala MJ (2008) Similar metabolic adaptations during exercise after low volume sprint interval and traditional endurance training in humans. J Physiol 586:151–160

Chen HC, Bandyopadhyay G, Sajan MP, Kanoh Y, Standeart M, Farese RV Jr, Farese RV (2002) Activation of the ERK pathways and atypical protein kinase C isoforms in exercise- and aminoimidazole-4-carboxamide-1-beta-D-ribose (AICAR)-stimulated glucose transport. J Biol Chem 277:23554–23562

Crambert G, Füzesi M, Garty Karlish S, Geering K (2002) Phospholemman (FXYD1) associates with Na,K-ATPase and regulate its transport properties. Proc Natl Acad Sci USA 99:11476–11481

Dela F, Holten M, Juel C (2004) Effect of resistance training on Na, K pump and Na^+/H^+ exchange protein densities in muscle from

control and patients with type 2 diabetes. Pflugers Arch 447:928–933

Despa S, Bossuyt J, Han F, Ginsburg KS, Jia L-G, Kutchai H, Tucker AL, Bers DM (2005) Phospholemmen-phosphorylation mediates the β-adrenergic effects on Na/K pump function in cardiac myocytes. Circ Res 97:252–259

Feschenko MS, Stevenson E, Sweadner KJ (2000) Interaction of protein kinase C and cAMP-dependent pathways in the phosphorylation at the Na,K-ATPase. J Biol Chem 275:34693–34700

Galuska D, Kotova O, Barrès R, Chibalina D, Benziane B, Chibalin AV (2009) Altered expression and insulin-induced trafficking of Na^+-K^+-ATPase in rat skeletal muscle: effects of high-fat diet and exercise. Am J Physiol Endocrinol Metab 297:E38–E49

Gato M, Terada S, Kato M, Katoh M, Yokozeki T, Tabata I, Shimokawa T (2000) cDNA cloning and mRNA analysis of PGC-1α in epitrochlearis muscle in swimming-exercised rats. Biochem Biophys Res Commun 274:350–354

Jensen TE, Rose AJ, Jørgensen SB, Brandt N, Schjerling P, Wojtaszewski JF, Richter EA (2007) Possible CaMKK-dependent regulation of AMPK phosphorylation and glucose uptake at the onset of mild tetanic skeletal muscle contraction. Am J Physiol Endocrinol Metab 292:E1308–E1317

Jensen TE, Wojtascewski JFP, Richter EA (2009) AMP-activated protein kinase in contraction regulation of skeletal muscle metabolism: necessary and/or sufficient? Acta Physiol 196:155–174

Jørgensen SB, Treebak JT, Viollet B, Schjerling P, Vaulont S, Wojtaszewski JFP, Richter EA (2007) Role of AMPKα2 in basal, training-, and AICAR-induced GLUT4, hexokinase II, and mitochondrial protein expression in mouse muscle. Am J Physiol Endocrinol Metab 292:E331–E339

Juel C (2006) Training-induced changes in membrane transport proteins of human skeletal muscle. Eur J Appl Physiol 96:627–635

Juel C (2009) Na^+-K^+-ATPase in rat skeletal muscle: muscle fiber-specific differences in exercise-induced changes in ion affinity and maximal activity. Am J Physiol Regul Integr Comp Physiol 296:R125–R132

Kristensen M, Juel C (2010) Na^+, K^+-ATPase Na^+ affinity in rat skeletal muscle fiber types. J Membr Biol 234:35–45

Kristensen M, Rasmussen MK, Juel C (2008) Na^+-K^+ pump location and translocation during muscle contraction in rat skeletal muscle. Pflugers Arch 456:979–989

Leick L, Wojtascewski JFP, Johansen ST, Kiilerich K, Comes G, Hellsten Y, Hidalgo J, Pilegaard H (2008) PGC-1α is not mandatory for exercise- and training-induced adaptive gene responses in mouse skeletal muscle. Am J Physiol Endocrinol Metab 294:E463–E474

Leick L, Hellsten Y, Fentz J, Lyngby SS, Wojtaszewski JFP, Hidalgo J, Pilegaard H (2009) PGC-1α mediates exercise-induced skeletal muscle VEGF expression in mice. Am J Physiol Endocrinol Metab 297:E92–E103

Lifshitz Y, Lindzen M, Garty H, Karlish SJD (2006) Functional interactions of phospholemman (PLM) (FXYD1) with the Na^+, K^+-ATPase. J Biol Chem 281:15790–15799

Lin J, Wu N, Tarr PT, Zhang C-Y, Wu Z, Boss O, Michael LF, Pulgserver P, Isotani E, Olson EN, Lowell BB, Bassel-Duby R, Spiegelman BM (2002) Transcriptional co-activator PGC-1α drives the formation of slow-twitch muscle fibers. Nature 418:797–801

Lin J, Wu PH, Tarr PT, Lindenberg KS, St-Pierre J, Zhang CY, Mootha VK, Jäger S, Vianna CR, Reznick RM, Cui L, Manieri M, Donovan MX, Wu Z, Cooper MP, Fan MC, Rohas LM, Zavachi AM, Cinti S, Shulman GI, Lowell BB, Krainc D (2004) Defect in adaptive energy metabolism with CNS-linked hyperactivity in PGC-1alpha null mice. Cell 119:121–135

Olesen J, Kiilerich K, Pilegaard H (2010) PGC-1α-mediated adaptations in skeletal muscle. Pflugers Arch 460:153–162

Pilegaard H, Saltin B, Neufer PD (2003) Exercise induces transient transcriptional activation of the PGC-1alpha gene in human skeletal muscle. J Physiol 546:851–858

Terada S, Goto M, Kato M, Kawanaka K, Shimokawa T, Tabata I (2002) Effects of low-intensity prolonged exercise on PGC-1α mRNA expression in rat epitrochlearis muscle. Biochem Biophys Res Commun 296:350–354

Vadasz I, Dada LA, Briva A, Trejo HE, Welch LC, Chen J, Toth PT, Lecuona E, Witters LA, Schumacher PT, Chandel NS, Seeger W, Sznajder JI (2008) AMP-activated protein kinase regulates CO2-induced alveolar epithelial dysfunction in rat and human cells by promoting Na,K-ATPase endocytosis. J Clin Invest 118:752–762

Winder WW, Hardie DG (1996) Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. Am J Physiol Endocrinol Metab 270:E299–E304

Winder WW, Taylor EB, Thomason DM (2006) Role of AMP-activated protein kinase in the molecular adaptations to endurance exercise. Med Sci Sports Exerc 38:1945–1949

Zhang L, NG Y-C (2007) Fiber specific differential phosphorylation of the α1-subunit of the Na^+, K^+-ATPase in rat skeletal muscle: the effect of aging. Mol Cell Biochem 303:231–237

Zheng D, Perianayagam A, Lee DH, Brannan MD, Yang LE, Tellalian D, Chen P, Lemieux K, Marette A, Youn JH, McDonough AA (2008) AMPK activation with AICAR provokes an acute fall in plasma [K^+]. Am J Physiol Cell Physiol 294:C126–C135