Hypertrophy, increased ejection fraction, and reduced Na-K-ATPase activity in phospholemman-deficient mice

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Jia, Li-Guo, Claudia Donnet, Roberta C. Bogaev, Rebecca J. Blatt, Cindy E. McKinney, Kathleen H. Day, Stuart S. Berr, Larry R. Jones, J. Randall Moorman, Kathleen J. Sweadner, and Amy L. Tucker. Hypertrophy, increased ejection fraction, and reduced Na-K-ATPase activity in phospholemman-deficient mice. Am J Physiol Heart Circ Physiol 288: H1982–H1988, 2005. First published November 24, 2004; doi:10.1152/ajpheart.00142.2004.—Phospholemman (PLM) is a single-span membrane protein abundantly expressed in the cardiac and skeletal sarcolemmal membrane (4, 10, 11, 37) that was initially identified and characterized as a major substrate for PKA and PKC in membrane (4, 10, 11, 37) that was initially identified and abundantly expressed in the cardiac and skeletal sarcolemma. Biochemical, cellular, and electrophysiological studies have suggested a number of possible roles for this protein, including ion channel modulator, taurine-release channel, Na+/Ca2+ exchanger modulator, and Na-K-ATPase-associated subunit. We have generated a phospholemman-deficient mouse. The adult null mice exhibited increased cardiac mass, larger cardiomyocytes, and ejection fractions that were 9% higher by magnetic resonance imaging compared with wild-type animals. Notably, this occurred in the absence of hypertension. Total Na-K-ATPase activity was 50% lower in the phospholemman-deficient hearts. Expression (per unit of membrane protein) of total Na-K-ATPase was only slightly diminished, but expression of the minor α2-isofom, which has been specifically implicated in the control of contractility, was reduced by 60%. The absence of phospholemman thus results in a complex response, including a surprisingly large reduction in intrinsic Na-K-ATPase activity, changes in Na-K-ATPase isofom expression, increase in ejection fraction, and increase in cardiac mass. We hypothesize that a primary effect of phospholemman is to modulate the Na-K-ATPase and that its reduced activity initiates compensatory responses.

FXYD protein family; heart; mouse; knockout

PHOSPHOLEMMAN (PLM) is a single-span membrane protein abundantly expressed in the cardiac and skeletal sarcolemmal membrane (4, 10, 11, 37) that was initially identified and characterized as a major substrate for PKA and PKC in α- and β-adrenergic stimulation (15, 28). PLM is a substrate for multiple protein kinases (16, 27, 36), raising the possibility that it integrates signals from multiple pathways. The function of PLM is not known, but its expression induces anion currents in Xenopus oocytes (13, 24) and increases taurine efflux (22, 25, 26) and regulatory volume decrease in cultured cells (23, 25).

In lipid bilayers, reconstituted PLM forms channels that are selective for zwitterions (12, 22). Most recently, PLM has been shown to be an accessory protein for the Na-K-ATPase (the sodium pump) and to modify its activity (5, 7).

PLM is a member of the FXYD protein family. FXYD proteins have a single transmembrane domain and a 35-amino acid signature motif that includes seven invariant amino acids, starting with PFXYD (34). Of the seven identified mammalian FXYD proteins, four have been shown to regulate the Na-K-ATPase, including the γ-subunit (1, 29, 35), CHIF (FXYD4) (2), FXYD7 (3), and PLM (5). Additionally, a phosphorylatable FXYD protein from the shark rectal gland (a salt-secreting organ) coimmunoprecipitates with Na-K-ATPase and affects its activity (17). Hence, there is a convincing body of evidence linking PLM and the other members of the FXYD family to regulation of Na-K-ATPase. To further test the function of PLM in vivo, we have generated a PLM-deficient mouse.

MATERIALS AND METHODS

Animal care. The mice used in the experiments were housed in a vivarium supervised by the Department of Comparative Medicine at the University of Virginia Health Science Center. Standard care was given to all mice used for PLM experiments. All protocols applied to the mice in this study were approved and supervised by the Animal Care and Use Committee at the University of Virginia.

Targeting the phospholemman gene. A mouse line deficient in PLM was generated by replacing portions of the PLM gene in the AB 2.2 stem cell line derived from 129/SvJ mice. The mouse PLM gene was cloned from a 129/SvJ mouse lambda genomic DNA library (Stratagene, La Jolla, CA) as previously reported (4). The targeting construct was composed of 5’ and 3’ homology domains flanking a 5.0 kb insert containing lacZ with a nuclear localization sequence (3.4 kb) and a neomycin resistance gene (1.6 kb) ligated into the pKO cloning vector (Lexicon Genetics, Woodlands, TX) (Fig. 1). The insert replaced the sequence of the PLM gene from exon 3 through exon 5. The 5’ homology domain was a 1466 bp Hpal/Ncol fragment. The 3’ homology domain was a 1697-bp Bpu1102 fragment. A diptheria toxin A subunit (DTA) gene was incorporated into the vector outside of the homology domains. Targeted stem cells were selected and genotyped using PCR and Southern blotting. PCR of the 5’ homology domain generated a wild-type fragment of 584 bp and targeted fragment of 213 bp. Amplicons from PCR of the 3’ homology

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domain included a wild-type product of 1077 bp and a targeted product of 720 bp. 5' Southern blotting was performed following genomic DNA digestion with DraI (5' analysis) or Accl/EcoRV (3' analysis) resulting in 5' fragments of 3.4 kb (wild type) or 5.0 kb (targeted) or 3' fragments of 2.5 kb (wild type) or 4.3 kb (targeted), respectively. The 5' probe was a PCR fragment, including base pairs 3902–5062 of the murine PLM gene inside the 3' homology domain. Targeted stem cell lines were karyotyped and screened for pathogens. Blastocyst injection and generation of germ-line chimeric mice were performed in the Transgenic Facility at the University of Virginia. Agouti chimeric offspring were mated to C57BL/6 (Jackson Laboratory, Bar Harbor, ME) mice. The agouti offspring were genotyped, and the chimeric offspring were mated to C57BL/6 (Jackson Laboratory, Bar Harbor, ME) mice. The agouti offspring were genotyped, and the chimeric offspring were mated to C57BL/6 (Jackson Laboratory, Bar Harbor, ME) mice. The agouti offspring were genotyped, and the chimeric offspring were mated to C57BL/6 (Jackson Laboratory, Bar Harbor, ME) mice.

Murine cardiac magnetic resonance imaging. PLM+/+ and PLM−/− littersmates over 3 mo in age were studied by cardiac magnetic resonance imaging (MRI) to determine ejection fraction by using methods developed previously (30). For imaging, the mice were anesthetized with a mixture of 1% isoflurane and oxygen continuously administered at 0.2 l/min via nose cone. Imaging was performed on a Varian 200/400 Inova 4.7T MRI system with Magnex gradients (80 G/cm maximum strength) and a custom-built 2.5-cm quadrature birdcage radiofrequency coil (RF Design Consulting, Newbury, FL). Acquisition of MRI data was gated to the ECG, and body temperature was maintained at 37°C. Short-axis cine images were acquired using an ECG-triggered two-dimensional (2D) gradient echo sequence. The echo time was 3.9 ms, and the repetition time value was continuously adjusted (5.0–10.0 ms) to obtain 14–16 equally spaced phases during each cardiac cycle depending on heart rate. A 30° flip angle was used. A 2.56-cm × 2.56-cm field of view was acquired with a matrix of 320 × 320 yielding a final resolution of 100 × 100 μm². Three signal averages were used, resulting in an acquisition time for each slice of about 4 min. Six to eight 1-mm thick slices were obtained to cover the entire heart. Images were quantitatively analyzed using the ARGUS image analysis program (Siemens Medical Systems, Princeton, NJ). Endocardial contours were manually traced by a blinded image analyst at the end diastolic and the end systole for each slice. The left ventricular (LV) diastolic volume (EDV), end-systolic volume (ESV), and ejection fractions were computed using Simpson’s rule.

Histology. Paraffin-embedded mouse hearts were mounted, sectioned, and stained with hematoxylin and eosin. Photographic images of whole heart sections and cardiac myocytes were digitally captured using the Image-Pro program (Media Cybernetics, Hercules, CA).
**Immunoprecipitation of PLM with Na-K-ATPase.** To immunoprecipitate Na-K-ATPase, we used a monoclonal antibody αk against the α-subunit (Developmental Studies Hybridoma Bank). Pig cardiac sarclemma was isolated by sucrose gradient centrifugation (9). Two milligrams of protein were solubilized in 6 M guanidinoacetic acid containing 0.5% sodium dodecyl sulfate (C12E8, Calbiochem, San Diego, CA) for 10 min at room temperature in 2 ml of buffer containing (in mmol/l) 140 NaCl, 25 imidazole, and 1 and EDTA; pH 7.3. The extract was diluted with an equal volume of detergent-free buffer, and insoluble material was sedimented by centrifugation for 30 min at 20,000 g at 4°C. Aliquots of the starting material, pellet (resuspended in the same volume of buffer), and supernatant were saved to evaluate solubilization. The supernatant was divided and incubated with primary antibodies or control IgG (1–2 μg/ml) overnight at 4°C. After 2 h incubation with 40 μl of secondary goat anti-rabbit or goat anti-mouse IgG antibodies covalently bound to agarose beads (Sigma-RBI, St. Louis, MO), the immunoprecipitates were collected by centrifugation at 9,300 g for 10 min at 4°C and washed four times with solubilization buffer containing 0.05% C12E8. The final wash, the pellets were resuspended in 40 μl of ×1 electrophoresis sample buffer. Samples were incubated for 20 min at room temperature and centrifuged at 9,300 g for 10 min. Supernatants were saved. Pellets were washed with an additional 20 μl of electrophoresis sample buffer and centrifuged again. The supernatants were combined and heated for 10 min at 65°C to dissociate IgG before loading on the gel. Electrophoresis was on 12.5% polyacrylamide Tricine gels (31). The Na-K-ATPase α-subunit was detected with polyclonal antibody K1 (raised against dog kidney αk), and PLM was detected using affinity-purified antibodies against the COOH-terminus of phospholemman, PLM-C1 (24).

**Na-K-ATPase activity.** A crude sarcolemma fraction was isolated from mouse hearts. A pool of 5–10 hearts was minced and homogenized in a buffer containing (mmol/l) 20 Tris, 1 EDTA, and 0.315 sucrose; pH 7.5. The homogenates were centrifuged at 40,000 rpm in a Ti45 rotor (Beckman, San Antonio, TX). Pellets were collected, rehomogenized in the same buffer, and centrifuged again. The new pellets were resuspended and layered on a sucrose-step gradient (in mol/l: 0.75, 0.9, 1.2, 1.4, in 20 Tris, 1 EDTA; pH 7.5) and centrifuged at 27,000 rpm for 6 h. The sarcolemma-enriched fractions containing part of exon 3 and all of exons 4 and 5 (Fig. 1A) were incubated for 20 min at room temperature and centrifuged at 9,300 g for 10 min. Supernatants were saved. Pellets were washed with an additional 20 μl of electrophoresis sample buffer and centrifuged again. The supernatants were combined and heated for 10 min at 65°C to dissociate IgG before loading on the gel. Electrophoresis was on 12.5% polyacrylamide Tricine gels (31). The Na-K-ATPase α-subunit was detected with polyclonal antibody K1 (raised against dog kidney αk), and PLM was detected using affinity-purified antibodies against the COOH-terminus of phospholemman, PLM-C1 (24).

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**RESULTS**

The development of phospholemman-deficient mice. We interrupted the PLM gene with the in-frame insertion of a lacZ coding sequence containing a nuclear localization sequence (nls) resulting in the deletion of a region of the PLM gene containing part of exon 3 and all of exons 4 and 5 (Fig. 1A). This region encodes 36 of the 72 nonsignal peptide amino acids in PLM, including the entire transmembrane domain. The lacZ gene is spliced at an NcoI site in-frame with the first 20 amino acids in PLM. G418 was used for positive selection and DTA to reduce falsely positive stem cells. Over 400 neomycin-resistant stem cells were screened. One positive clone was obtained that demonstrated homologous recombination by PCR and Southern blot analyses, was pathogen-free, and had a normal karyotype. This clone was amplified and used for blastocyst injection. Six of 13 chimeric mice demonstrated germ line transmission of the targeted gene. Offspring from heterozygous breeding pairs were genotyped using 5′ and 3′ PCR and Southern blotting strategies. Figure 1B shows 5′ PCR confirmation that the PLM gene was targeted, and Fig. 1C represents the confirmatory 5′ Southern blot assay performed under high stringency conditions. Animals were also genotyped using 3′ PCR and 3′ Southern blot assays (data not shown). Additionally, PCR products were subcloned and sequenced to confirm homologous recombination of both 5′ and 3′ homology domains.

Samples of crude heart homogenate were used for Western blot analysis (Fig. 1D). Lane 1 demonstrates the presence of PLM protein in a PLM+/+ mouse, and lanes 2 and 3 from PLM−/− mice show no protein. Homogenates from skeletal muscle and liver gave similar results (data not shown).

Genomic DNA analysis and absence of protein expression indicate that we have successfully targeted the PLM gene. Both heterozygous and homozygous mice reproduce normally and appear healthy up to at least 104 wk of age.

MRI imaging shows increased ejection fractions in PLM−/− mice. The hearts of 10 PLM−/− and 9 PLM+/+ mice were imaged using MRI to determine whether or not there was a structural phenotype caused by phospholemman deficiency. Hearts from both groups were structurally normal, but PLM−/− mice had higher ejection fractions than PLM+/+ mice (73 ± 2% vs. 64 ± 3%, P = 0.017, Student’s t-test) (Fig. 2). In a separate cohort of age-matched littermates scanned serially at 2 and 5 mo of age, the differences in ejection fraction between the two groups did not become apparent until the animals were 5 mo of age (data not shown).

**Increased mass of PLM−/− hearts.** The cardiac mass-tobital length ratios are given in Table 1. PLM−/− animals have significantly higher cardiac mass-tobital length ratios compared to controls. Whether the differences are due to cardiac hypertrophy or to changes in body weight is being investigated. The cardiac masses of PLM−/− and PLM+/+ mice were measured by MRI imaging, and the results are shown in Table 1. The cardiac masses of the PLM−/− mice were significantly higher than those of the PLM+/+ mice, with a 10.2% difference. The differences were statistically significant (P < 0.017, Student’s t-test) (Fig. 2). In a separate cohort of age-matched littermates scanned serially at 2 and 5 mo of age, the differences in ejection fraction between the two groups did not become apparent until the animals were 5 mo of age (data not shown).
a 24% larger ratio than their wild-type counterparts. Figure 3 shows representative photomicrographs of heart tissue taken from the left ventricles of PLM+/+ (Fig. 3A) and PLM−/− mice (Fig. 3B). The cardiomyocytes of PLM−/− animals appeared larger than PLM+/+ cardiomyocytes, and this was borne out in counts of nuclei per unit area in 36 representative microscope fields from each genotype, depicted in Table 1 as calculated counts of nuclei per unit area in 36 representative microscope fields from each genotype, depicted in Table 1 as calculated cell size. Additional evidence supporting the observed increase in cardiomyocyte size in PLM−/− mice comes from the comparison of the cell membrane capacitance from isolated myocytes (data not shown).

**Murine blood pressures are not affected by PLM deficiency.** There was no significant difference in systolic tail-cuff blood pressure between PLM+/+ and PLM−/− mice. The means were 115 ± 3 mmHg for PLM+/+ and 120 ± 2 mmHg for PLM−/− animals (n = 9 PLM+/+, 10 PLM−/−, P = 0.26). We conclude that the increased cardiac mass in PLM−/− mice is not due to hypertension.

**PLM associates with Na-K-ATPase in cardiac sarcolemma.** Recent evidence suggests that PLM can associate in a complex with the α- and β-subunits of the Na-K-ATPase (5, 7). Whereas this was observed for bovine cardiac sarcolemma before, it was without controls for the solubilization of the enzyme complex and the specificity of the immunoprecipitation. Here we confirm the association of PLM with Na-K-ATPase in a sarcolemma fraction isolated from pig heart. Pig heart Na-K-ATPase contains only the α1-subunit, not too dissimilar from the mouse heart Na-K-ATPase, which contains predominantly the α1-subunit. Figure 4 shows the presence of Na-K-ATPase α-subunit and PLM in sarcolemma starting material and the detergent-solubilized fraction. The pellet of the detergent extraction step had some α-subunit but negligible PLM. Immunoprecipitation was performed with an antibody specific for the Na-K-ATPase α-subunit and with nonimmune IgG as a control.

**Na-K-ATPase activity is decreased in PLM−/− hearts.** We tested the hypothesis that Na-K-ATPase activity was altered in the PLM−/− animals. ATP hydrolysis was measured in vitro with a sarcolemma-enriched membrane fraction as the ouabain-sensitive fraction of the total generation of Pi. Na-K-ATPase activity in sarcolemma without PLM was only 40% of that in the wild-type sarcolemma (Table 2). To correct for any change in total Na-K-ATPase in the samples from hypertrophied hearts, Western blot analyses were stained for the catalytic subunit α, with an antibody that detects all α-isoforms, and quantified by scanning. A representative blot is shown in Fig. 5A. Western blot analyses were also used to determine whether sarcolemma-enriched membrane fractions from PLM-deficient hearts had any change in the composition of Na-K-ATPase subunits. A representative blot is shown in Fig. 5B, and the averaged data are in Table 3. Hypertrophy can in principle result in a real reduction in enzyme expression, or an apparent reduction (if expression is constant but expression of other membrane components is increased, or if contamination of sarcolemma by other cellular fractions increases). To evaluate the large observed loss of Na-K-ATPase activity, it is important to correct for any reduction in Na-K-ATPase protein per unit of sample assayed. On average the reduction of total Na-K-ATPase protein in PLM knockout hearts was ~20%, and there was no statistically significant difference in the levels detected by different antibodies (the α-isoform pan-specific

**Table 1. Relative mouse heart mass and cell size**

<table>
<thead>
<tr>
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<th>PLM+/+</th>
<th>n</th>
<th>PLM−/−</th>
<th>n</th>
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<tbody>
<tr>
<td>Heart/body weight, mg/g</td>
<td>4.92±0.22</td>
<td>11</td>
<td>5.46±0.21</td>
<td>10</td>
</tr>
<tr>
<td>Heart weight/tibia, mg/mm</td>
<td>6.68±0.37</td>
<td>11</td>
<td>8.25±0.62*</td>
<td>10</td>
</tr>
<tr>
<td>Calculated myocyte area, μm²</td>
<td>333.9±17.44</td>
<td>9</td>
<td>388.7±16.26*</td>
<td>9</td>
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Data are means ± SE n, number mice. *P ≤ 0.05.

**AJP-Heart Circ Physiol • VOL 288 • APRIL 2005 • www.ajpheart.org**

**Fig. 3. Hematoxylin plus eosin-stained PLM+/+ (A) and PLM−/− (B) heart tissue. Cardiomyocytes from PLM−/− mice are larger than from PLM+/+ controls. Photographs were taken at ×400.**
The dramatic reduction of pump activity with a marginal decrease in protein level suggests that PLM is a major regulator of Na-K-ATPase activity in hearts. This is probably mediated through direct physical interaction with the αβ-complex, illustrated by coimmunoprecipitation, although further PLM-mediated events such as recruitment of additional regulatory molecules may also occur. Because of the difficulty in isolating adequate quantities of sarcolemmal vesicles from the mouse heart for coimmunoprecipitation studies, we have used the pig heart to demonstrate the interaction between PLM and Na-K-ATPase in the sarcolemma. Ideally, these experiments would have been performed using mouse sarcolemma. We understand that there may be interspecies differences in the affinity of

antibody and those for α1- and β1-isoform, the major forms present). A reduction in α2 expression, on the other hand, was significant, although it is a minor component of the total enzyme activity. We also evaluated expression of α3 (the neonatal form), but it was undetectable in either wild-type or knockout mice. After correction for the 20% reduction in total α content, the Na-K-ATPase activity per unit of α-subunit was still reduced to 47% of the wild type. This is an apparent reduction in the turnover rate of the enzyme, apart from any change in the total amount of protein present in the heart or sarcolemma.

**DISCUSSION**

Using gene targeting, we have generated PLM-deficient mice with a phenotype of modest hypertrophy, independent of a rise in blood pressure, and small increase in ejection fraction. We did not observe severe hypertrophy or disorganization of myofibrils as is found in hypertrophic cardiomyopathy from the mutations in sarcomeric proteins. The phenotype appears more similar to models with compensatory increases in mass, such as pressure-overload models of hypertrophy or overexpression of certain proteins in the adrenergic signaling cascade. An important caveat to this study is that it was performed using mice with a mixed C57BL/6 129/SvJ genetic background. We have subsequently generated congenic animals backcrossed to the C57BL/6 strain, and preliminary results suggest that there may be phenotypic variation from the mixed animals.

Because PLM and other FXYD proteins interact with Na-K-ATPase, we measured Na-K-ATPase activity in PLM-deficient cardiac sarcolemma and found apparent maximal velocity (V_{max}) reduced by 50%. Although others (5) have observed that PLM reduces the apparent Na^+ affinity of the pump current in *Xenopus* oocytes, in preliminary experiments we did not see a marked difference in apparent Na^+ affinity between wild-type and PLM-deficient sarcolemmal Na-K-ATPase activity measured in vitro. Treatment of PLM-containing choroid plexus Na-K-ATPase with anti-PLM antibodies also results in a reduction in total activity, without an effect on apparent Na^+ affinity (7). The biochemical mechanisms of the effect of PLM and the consequences of differences in its phosphorylation levels in different tissues remain to be resolved, but the present data suggest that V_{max} effects predominate in a native tissue context.

**Table 2. Sarcolemma Na-K-ATPase activity of PLM−/− mice as percentage of activity in wild-type mice**

<table>
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<th>Activity, %</th>
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<tr>
<td>Na-K-ATPase/mg protein</td>
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<tr>
<td>Na-K-ATPase corrected for α-band density</td>
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Ouabain-sensitive hydrolysis of ATP by gradient-isolated cardiac sarcolemma membrane fractions from PLM+/+ and PLM−/− mice was measured with a test tube assay. Specific activities of the preparations (ouabain-sensitive ATPase activity) were typically 15–30 μmol ATP hydrolyzed per hour per milligram protein. n, Number of independent experiments. In three of the four experiments, the expression of Na-K-ATPase per milligram protein was assessed by measuring the band density for the α-subunit stained with a polyclonal antibody (KETYY) that does not discriminate between α1- and α2-isoforms, which revealed a small reduction in Na-K-ATPase level in the knockout sarcolemma fraction compared with wild-type (see also Table 3). This was used to correct the calculated fraction of activity found in the knockout samples so that the value of approximately 50% represents activity per unit of Na-K-ATPase α-subunit.

**Fig. 5. Western blot analysis of Na-K-ATPase and PLM content in wild-type and PLM knockout mice.** A: representative blot showing the contents of total Na-K-ATPase α-subunit and PLM in sarcolemma samples, as performed for the data of Table 2. The α-subunit was detected with anti-KETYY, and PLM with PLM-C2. B: representative blot showing the detection of Na-K-ATPase α1-, α2-, and β1-subunits in sarcolemma from wild-type and PLM knockout hearts, as performed for the data of Table 3. Lane 1, wild-type α1; Lane 2, PLM knockout α1; Lane 3, wild type stained for both α2 and β1; Lane 4, PLM knockout stained for both α2 and β1.
Ca\textsuperscript{2+}ility of cardiac myocytes is acutely controlled by cytoplasmic space. The classic effect of digitalis is to produce higher intracellular Na\textsuperscript{+} and thus reduce Ca\textsuperscript{2+} efflux via NCX1. This leads to better loading of sarcoplasmic reticulum Ca\textsuperscript{2+} stores and enhanced contractility (18). Hence, the increased ejection fraction in PLM-deficient mice would be predicted based on the reduced Na-K-ATPase activity.

Interestingly, overexpression of PLM in adult rat cardiac myocytes has been shown to acutely alter contractility as a function of extracellular Ca\textsuperscript{2+} (33). A rectification of this effect was observed when NCX1 was also overexpressed, leading to the novel hypothesis that PLM inhibits NCX1 (39). Consistent with these observations, downregulation of PLM resulted in alterations in contractility opposite to those observed with overexpression (20). Hypothetically, if PLM activates the Na-K-ATPase, it would functionally oppose the Na\textsuperscript{+} and Ca\textsuperscript{2+} gradient changes supported by NCX1, and this may be enough to explain the effects observed with alterations in PLM expression. However, modulation of contractility may also result from direct interaction between PLM and NCX1. PLM and NCX1 colocalize at the sarcolemma and in t-tubules (39), and coimmunoprecipitation experiments are consistent with interaction between the two proteins (20). Na-K-ATPase shares the same anatomic distribution (19, 21) as NCX1. In fact, disruption of the distribution of the Na-K-ATPase and NCX1 of the sarcoplasmic reticulum t-tubule junctional complex in mice deficient in ankyrin-B (21) suggests that PLM, Na-K-ATPase, and NCX1 could all be part of a multiprotein complex anchored together for optimal function. Their mutual interaction could be a matter of considerable importance.

From an entirely different perspective, the sodium pump has been implicated in signal transduction cascades resulting in altered gene expression independent of ion transport and leading to cellular growth. Xie and coworkers (38) have proposed that, following interaction with ouabain, Na-K-ATPase interacts with neighboring membrane proteins to trigger several signal transduction pathways, including activation of Src kinase, Ras, and p42/44 mitogen-activated protein kinases. In cardiomyocytes, protein synthesis and cellular hypertrophy are the outcome. Whether activation of a program of ventricular hypertrophy can be elicited by reducing Na-K-ATPase activity by other means is uncertain. Studies of mice engineered to have only one copy of the Na-K-ATPase \( \alpha_1 \) gene suggest that reduced enzymatic activity of Na-K-ATPase alone is not sufficient (8). Hearts from \( \alpha_1 \) heterozygotes with 60% of the \( \alpha_1 \)-isoforms to total Na-K-ATPase activity were hypocontractile and had normal size and histology. This contrasts with the 80% level of \( \alpha_1 \) protein and <50% of Na-K-ATPase activity in PLM-deficient hearts that show increased ejection fraction and mass.

In contrast, mouse hearts containing only one copy of the \( \alpha_2 \) gene showed 50% reduction in \( \alpha_2 \) protein without a significant reduction in total Na-K-ATPase activity, and they were hypercontractile, but also with normal size and histology (8). The opposite phenotypic responses of the \( \alpha_1 \) and \( \alpha_2 \) heterozygotes, which did not correlate with total remaining Na-K-ATPase activity, points to specific contributions of the \( \alpha \)-isoforms to the control of contractile performance. Although PLM-deficient mice showed reduction in expression of both \( \alpha_1 \)- and \( \alpha_2 \)-isoforms, there was a relatively greater reduction of \( \alpha_2 \), a result that might have thus contributed to the observed increases in ejection fraction.

### Table 3. Na-K-ATPase \( \alpha \)- and \( \beta \)-subunit reductions in hypertrophied sarcolemma of PLM knockout mice

<table>
<thead>
<tr>
<th>Antibody</th>
<th>n</th>
<th>PLM\textsuperscript{+/+}</th>
<th>PLM\textsuperscript{-/-}</th>
<th>Corrected†</th>
</tr>
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<tbody>
<tr>
<td>Pan-( \alpha )</td>
<td>2</td>
<td>100</td>
<td>81.0±18.0 (81%)</td>
<td></td>
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<tr>
<td>( \alpha_1 )</td>
<td>4</td>
<td>100</td>
<td>70.3±9.5 (77.7%)</td>
<td></td>
</tr>
<tr>
<td>( \alpha_2 )</td>
<td>4</td>
<td>100</td>
<td>35.8±8.6 (39.6%)</td>
<td></td>
</tr>
<tr>
<td>( \beta_1 )</td>
<td>2</td>
<td>100</td>
<td>90.5±9.1 (100%)</td>
<td></td>
</tr>
</tbody>
</table>

Sarcolemma fractions were isolated by centrifugation on sucrose gradients and approximately equal amounts of proteins were loaded onto SDS gels. Relative amounts of each subunit were assessed by densitometry of Western blot analysis stained with specific antibodies. Amido Black stain of the blots was used to correct for some differences in the amount of protein loaded. n, Number of independent experiments performed. Hypertrophied knockout hearts displayed a modest reduction in the major \( \alpha_1 \)- and \( \beta_1 \)-components and a larger reduction in the minor \( \alpha_2 \)-component.

\( \alpha \)-isoforms for PLM but propose that the existence of an interaction between PLM and Na-K-ATPase in the sarcolemma can be extrapolated to occur in both species. The direct and stable association of the \( \gamma \)-subunit with the \( \alpha \beta \)-complex of the kidney enzyme is very well characterized, and it is analogous to the association of the small hydrophobic regulatory protein phospholamban with sarcoplasmic reticulum Ca\textsuperscript{2+}, the Ca\textsuperscript{2+}-ATPase of cardiac sarcoplasmic reticulum (6, 32). Other evidence that PLM interacts directly with Na-K-ATPase comes from its copurification when Na-K-ATPase is isolated from the choroid plexus (7). That PLM substitutes for the \( \gamma \)-subunit of the Na-K-ATPase (FXYD2) in myocardium is an appealing hypothesis, because the \( \gamma \)-subunit itself has not been detected in the heart. The growing body of evidence that members of the FXYD family serve as regulatory subunits of the Na-K-ATPase indicates that they show distinct functional effects on its kinetic properties, allowing for tissue- and site-specific regulation (6). The functional effects of a given FXYD protein may also vary with the \( \alpha \)- and \( \beta \)-isoforms with which it associates, adding additional potential for diversity in Na-K-ATPase properties to meet particular tissue requirements. The phosphorylation sites of PLM, absent in the \( \gamma \)-subunit, allow for potential regulation by protein kinases, which may be important for the modulation of Na-K-ATPase function in response to adrenergic stimulation.

Whether the reduction in Na-K-ATPase activity is the primary cause of the increased mass and ejection fraction observed in PLM-deficient mouse hearts merits discussion. Other suspected roles for PLM such as induction of channel activity (13, 24), direct interaction with other ion exchangers (20, 33, 39), and response to insulin (36) have not been ruled out. It is possible that the observed decreases in Na-K-ATPase activity and expression are actually secondary to hypertrophy, which could be elicited in PLM deficiency by some other mechanism. Several hypertrophy models in various mammalian species show alterations in Na-K-ATPase activity, mostly reductions. In rat pressure-overload models, this has been accompanied by reductions in \( \alpha_2 \)-isoform expression as seen here (18). However, there are reasons to hypothesize that a primary reduction in Na-K-ATPase activity, caused by PLM deficiency, could have both acute and long-term effects on the cardiac phenotype. Reduction of Na-K-ATPase function may be associated with increases in contractility in a manner analogous to those seen with ouabain or digitalis inhibition of the pump. Contractility of cardiac myocytes is acutely controlled by cytoplasmic Ca\textsuperscript{2+} redistribution between internal stores and extracellular space.
In summary, we prepared mice deficient in PLM to gain insight into the function of this small membrane phosphoprotein. Our major findings are that the mice have mild hypertrophy in the absence of hypertension or severe cardiomyopathy, and that Na-K-ATPase activity is reduced. We interpret the results as further evidence of functional interaction between PLM and Na-K-ATPase.

REFERENCES


