Phospholemman overexpression inhibits Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in adult rat cardiac myocytes: relevance to decreased Na\textsuperscript{+} pump activity in postinfarction myocytes

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Phospholemman (PLM) is a 72-amino acid membrane phosphoprotein with a single transmembrane domain (24). It belongs to the FXYD gene family of small ion transport regulators (36). Studies in noncardiac tissues suggest that PLM can be a channel (15), a channel subunit, or an ion transport regulator (4, 9, 21–23) and is likely involved in regulation of cell volume (7, 22, 23). In heart and skeletal muscle, PLM is a major sarcolemmal substrate for protein kinase A (PKA) and protein kinase C (PKC) (16, 24, 25). Specifically, β-adrenergic agonists phosphorylate serine\textsuperscript{68} via PKA whereas PKC phosphorylates both serine\textsuperscript{68} and serine\textsuperscript{63} at the COOH terminus of PLM (40). Additional studies by Crambert et al. (6) and Feschenko et al. (9) demonstrated association of PLM with α-subunits of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in bovine cardiac sarcolemma and central nervous system. When coexpressed with α- and β-subunits of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in Xenopus oocytes, PLM was shown to modulate Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity, primarily by decreasing apparent affinities for Na\textsuperscript{+} and K\textsuperscript{+} without affecting V\textsubscript{max} (6). It is not known whether PLM directly affects Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in cardiac myocytes.

In cardiac sarcolemma isolated from the uninfarcted portion of rat left ventricles 8–16 wk after myocardial infarction (MI), Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activities were depressed primarily because of decreases in V\textsubscript{max} without any changes in the apparent affinities for Mg-ATP, Na\textsuperscript{+}, and K\textsuperscript{+} (8). In addition, in rat hearts subjected to coronary ligation, application of cDNA microarrays (containing 86 known genes and 989 unknown cDNAs) to analyze transcript levels indicated that PLM was one of only 19 genes to increase after MI (29). Although reduced expression of both α\textsubscript{1}- and α\textsubscript{2}- but not α\textsubscript{3}-isoforms of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase may account for the decreased V\textsubscript{max} post-MI (28, 30), increased PLM expression post-MI may also contribute to the suppression of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity. The present study was undertaken to test the hypothesis that enhanced PLM expression partly explains the depressed Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activities observed in post-MI rat hearts.

METHODS

Induction of myocardial infarction. To induce MI in male Sprague-Dawley rats (~250 g), the left main coronary artery of each anesthetized (2% isoflurane-98% O\textsubscript{2}), intubated, ventilated rat, was ligated 3–5 mm distal to its origin from the ascending aorta (5, 41, 45). Sham operation was identical to MI, except that the coronary artery was not...
ligated. In our hands, sham-operated (Sham) rats had close to 100% survival whereas the mortality for coronary ligation procedure was ~30% within 24 h of the operation. All surviving rats (Sham, n = 3; MI, n = 6) received rat chow and water ad libitum and were maintained on a 12:12-h light-dark cycle. Survivors typically had 36 ± 3% of myocardium infarcted as determined histologically (5). In addition, despite no overt signs of heart failure in MI rats, we observed at 1 and 3 wk postinfarction 20% lower LV systolic pressure in MI hearts when perfused in vitro (5). At 3 and 7 days post-MI, MI rats were overdosed with pentobarbital sodium (34 mg/kg body wt ip), and the left ventricles and septae were excised for immunoblotting studies. Sham-operated rat hearts were harvested at 7 days postoperatively for protein measurements. The protocol for induction of MI and subsequent heart excision was approved by Institutional Animal Care and Usage Committee.

Myocyte isolation and culture. Cardiac myocytes were isolated from the septum and left ventricular free wall of normal male Sprague-Dawley rats (~280 g), seeded on laminin-coated coverslips, and subjected to continuous pacing culture [1 Hz, extracellular Ca2+ concentration ([Ca2+]o) = 1.8 mM] as previously described (20, 32, 33, 35–37). Under continuous pacing culture conditions, we have previously demonstrated that myocyte contractility did not decline for at least 72 h (33). The protocol for heart excision for myocyte isolation was approved by Institutional Animal Care and Usage Committee.

Adenoviral infection of cardiac myocytes. Recombinant, replication-deficient adenovirus (Adv) expressing either green fluorescent protein (GFP) alone or GFP and dog PLM was constructed as described previously (33). Two hours after isolation, myocytes were infected with Adv-GFP or Adv-GFP-PLM and studied after 72 h of continuous pacing culture as previously described (20, 32, 33). We have previously demonstrated that over 95% of myocytes were successfully infected (44) and that adenoviral infection of myocytes had no effects on myocyte contractility when examined after 72 h of continuous pacing culture (33). We have also shown that the effects of PLM overexpression on contractility and intracellular Ca2+ concentration ([Ca2+]i) transients were manifest 72 h after Adv-PLM infection (33). In addition, PLM overexpression did not affect action potential amplitude and morphology, sarcoplasmic reticulum Ca2+ uptake, and protein levels of Na+/Ca2+ exchanger, α-subunits of Na+-K+-ATPase, and sarco(endo)plasmic reticulum Ca2+-ATPase (33). Two hours after isolation, myocytes were transfected with Adv-PLM or Adv-GFP and dog PLM cDNA. Myocytes were harvested 72 h post-transfection. PLM overexpression on contractility and intracellular Ca2+ transients were assessed as previously described (33). Two hours after isolation, myocytes were transfected with Adv-GFP or Adv-GFP-PLM and studied after 72 h of continuous pacing culture as previously described (20, 32, 33).

Measurement of Na+/K+-ATPase pump current. Whole cell patchclamp recordings were performed at 30°C as described previously (20, 32, 37, 42, 45). Standard pipette filling solution consisted of (in mM): 70 Na-aspartate, 20 K-aspartate, 8 CsOH, 20 K-aspartate, 8 CsOH, 7 MgSO4, 11 EGTA, 10 HEPES, 5 NaATP, and 0.2 GTP, pH 7.2. The Na+ concentration in the pipette ([Na+]pip) was varied between 5 and 80 mM by equimolar substitution of Na+ with Cs+. At 5 mM [Na+]pip, K2ATP was used instead of NaATP. External solution contained (in mM) 137.7 NaCl, 5.4 KCl, 2.3 NaOH, 1 MgCl2, 2 BaCl2, 1 CdCl2, 5 HEPES, and 10 glucose, pH 7.4. Extracellular K+ concentration ([K+]o) was varied between 1 and 18 mM.

Holding potential was switched from −70 to −40 mV (300 ms) before application of a negative voltage ramp (from +60 to −120 mV, 20 mV/s; Fig. 2B). Ouabain (1 mM) was added, and the voltage-ramp stimulus protocol was repeated. Currents were filtered at 1 kHz, and data were acquired at 2 kHz. Ip was defined as the difference in currents before and after ouabain addition. To facilitate comparison of Ip among cells, Ip of each myocyte was divided by its capacitance Cm to account for variations in cell sizes (42, 45).

To determine the concentration of [Na+]pip (Km,Na+) required to attain half-maximal Ip at each voltage, [K+]o was set to be saturating at 18 mM whereas [Na+]pip was varied between 5 and 80 mM. Similarly, to determine the [K+]o (Km,K+), required to attain half-maximal Ip, [Na+]pip was set at the saturation level of 80 mM while [K+]o was varied between 1 and 18 mM. Mean Ip values at each [Na+]pip or [K+]o were used to fit the Hill equation, using the Levenberg-Marquardt algorithm (SlideWrite Plus; Advanced Graphics Software, Encinitas, CA) which minimizes the sum of square deviation (χ2 value). Values for nH (the Hill coefficient), I0(max) (maximal Ip), and Km,Na+ or Km,K+ were determined at each voltage from −70 to +60 mV.

PLM, caselequestrin, and Na+–K+–ATPase immunoblotting. Left ventricles and septae were excised at 3 and 7 days post-MI and homogenized separately in a buffer described previously (44). Cultured myocytes were harvested for immunoblotting on day 3 as described previously (20, 32, 33, 37, 42–44). Primary antibodies used were as follows: for unphosphorylated PLM, C2 Ab (1:12,000) (27, 32, 33); for PLM phosphorylated at serine68, C68P Ab (1:10,000) (27, 31); and for caselequestrin, anti-caselequestrin antibody (1:250; Swant). Isoform-specific antibodies were used for α1-subunit (0.5 μg/ml; catalog no. 96-520, Upstate USA, Charlottesville, VA) and α2-subunit of Na+–K+–ATPase (1:1,000; polyclonal antibody gift from Dr. Robert Levenson, Pennsylvania State University).

Coimmunopurification experiments. C2 Ab was covalently linked to agarose support beads according to manufacturer’s instructions (Affi-Gel HZ immunofinity kit; catalog no. 153-6060, Bio-Rad) (20). Myocyte lysates were repeatedly (50 times) loaded onto the immunoaffinity column, which, after being washed, was eluted with 0.2 M glycine-HCl, pH 2.5, as previously described (20). The three eluant fractions (500 μl each) with the highest protein concentrations were subjected to PAGE (10% polyacrylamide gel, 32 to 40 μl/ lane), followed by protein transfer to ImmunoBlot PVDF membranes for detection of PLM and (α1 + α2)-subunits of Na+–K+–ATPase (α3-antibody, 1:250; Developmental Studies Hybridoma Bank, University of Iowa) by Western blotting.

Quantitative real-time RT-PCR studies. RNA (18S and 28S bands) from GFP and PLM myocytes were purified (RNeasy Mini Kit; Qiagen; Valencia, CA), visualized by chromatography (Agilent 2100 Bioanalyzer; Agilent Technologies) and concentrations adjusted by UV spectrophotometry. First strand cDNA was synthesized from 1.5 μg of total RNA by using oligo(dT)15 primers and random hexamer primers (SuperScript III Reverse Transcription kit; Invitrogen; Carlsbad, CA). Forty-two nanograms of cDNA per sample were used as a template for a real-time PCR, using a SYBR Green Master Mix (Qiagen) and gene-specific primers (GenScript; Scotts Plains, NJ). PCR amplification was performed (Sequence Detection System 7000; Applied Biosystems; Foster City, CA) with the following cycling parameters: 95°C for 10 min, followed by 45 cycles of 95°C for 15 s then 60°C for 1 min. Taqman 18S RNA primers (Eurogentec; San Diego, CA) were also used with 42 ng of cDNA and Taqman PCR Master Mix (Qiagen) under identical conditions for amplification of 18S, which was used as an internal standard. To exclude the possibility of genomic DNA contamination, control PCR reactions with no cDNA template were also performed for each gene-specific primer and probe set. Amplification data for α1- and α2-subunits of Na+–K+–ATPase were normalized for 18S within each individual PCR reaction. Duplicates of each PCR reaction were performed, and the resultant data were averaged. The following primers and probe set were utilized for real-time PCR amplification: for Na+–K+–ATPase α2-subunit (Atp1A2, ratus norvegicus; accession no. NM_012504) forward 5′-CAGCGCTTTCTTGTGCA-T3′, reverse 5′-TGGTTC TTGTTGATGTTCTCCTGCTG-3′ and probe 5′-Fam-TGCAGATGACCAAGT CAGCCGA-3′; for Na+–K+–ATPase α3-subunit (Atp1A1, ratus norvegicus; accession no. NM_012505) forward 5′-CACGGCTTCTTCGTGGATGAC-3′, reverse 5′-TCTTCTCTCCTGGATGAC-3′ and probe 5′-Fam-ACATGTTGCTCAGCAAGACTCGT-3′ Tamra.

Statistical analysis. All results are expressed as means ± SE. In experiments in which Ip was measured as a function of experimental group (GFP vs. PLM), voltage, and [Na+]pip or [K+]o, three-way ANOVA was performed to determine significance of difference.
were measured after 72 h of culture.

Student’s t-test was used to compare protein abundance between Sham and MI hearts and between GFP and PLM myocytes. A commercial statistical analysis package (JMP 4.04) was used. In all analyses, \( P < 0.05 \) was taken to be statistically significant.

**RESULTS**

**Effects of myocardial infarction on PLM expression in adult rat myocytes.** Figure 1 shows that at 3 and 7 days post-MI, PLM increased in MI myocytes compared with Sham myocytes. There were no differences in PLM protein levels between septum and left ventricle, and the results were combined. At 3 days post-MI, PLM increased 2.4-fold in MI myocytes (241 ± 53 vs. 110 ± 35 arbitrary units) (\( P = 0.066 \)). At 7 days post-MI, the fourfold increase in PLM in MI myocytes (407 ± 83 vs. 110 ± 35 arbitrary units) is statistically significant (\( P = 0.008 \)). Calsequestrin was used as an internal control for protein loading because its expression has been shown to be unchanged during ontogenic development, aging, cardiac hypertrophy, and failing human myocardium (11). There were no differences in calsequestrin levels between sham (292 ± 40 arbitrary units) and day 3 MI hearts (326 ± 24 arbitrary units) (\( P = 0.48 \)) and between sham and day 7 MI hearts (306 ± 26 arbitrary units) (\( P = 0.78 \)).

**Effects of PLM overexpression on Ip in adult rat myocytes.** Figure 2A shows that, in experiments in which GFP myocytes were voltage-clamped at \(-40 \text{mV}\), either reducing \([K^+]_\text{o}\) to zero in one myocyte (top) or adding 1 mM ouabain to another myocyte (bottom) resulted in decreases in steady-state membrane current, indicating that the \([K^+]_\text{o}\) or ouabain-sensitive currents represented Ip under our experimental conditions. The apparent differences in Ip measured under 0-[K\(^+\)]\(_o\) conditions and after ouabain addition were due to differences in cell sizes.

Figure 2B shows the voltage-ramp protocol applied to myocytes at 30°C. Figure 2C shows ramp currents measured in a control GFP myocyte (with \([\text{Na}^+]_\text{pip}\) and \([\text{K}^+]_\text{o}\) at 80 and 5.4 mM, respectively) in the absence and presence of 1 mM ouabain. The ouabain-sensitive currents, i.e., Ip, are displayed in Fig. 2D. It is clear that Ip was voltage dependent in that its amplitude was small at negative membrane potentials and progressively increased with depolarization, reaching a maximum at \(-0 \text{mV}\). In addition, the absolute magnitudes of Ip were generally lower in PLM than GFP myocytes (Fig. 2D). Two-way ANOVA confirmed a significant group (GFP vs. PLM) effect (\( P < 0.0001 \)). In both GFP and PLM myocytes, depolarization to more positive potentials increased the absolute magnitude of Ip (voltage effect, \( P < 0.0001 \)).

The relationship between Ip and \([\text{Na}^+]_\text{pip}\) (with \([\text{K}^+]_\text{o}\) saturating at 18 mM) measured at \(-10 \text{mV}\) is shown in Fig. 3A. The fitted values for \(k_{0.5,\text{Na}^+}\) are 9.95 ± 0.52 and 9.96 ± 0.73...
function of Na\(^{+}\) concentration of Na\(^{+}\)nHill coefficient (shown for GFP (broken line) and PLM (solid line) myocytes, from which the shown. Mean Ip values are fitted to the Hill equation, and the fitted curves are varied between 5 to 80 mM.

30°C was measured in GFP and PLM myocytes (METHODS). [Na\(^{+}\)]pip required to attain half-maximal Ip at each voltage (METHODS). [Na\(^{+}\)]pip was measured in 4 – 8 myocytes for each [Na\(^{+}\)]pip and Imax (shown for data relating mean Ip and [Na\(^{+}\)]pip from −70 to +60 mV are shown for control GFP (○) and PLM (□) myocytes.

When Ip was analyzed as a function of group (GFP vs. PLM), voltage, and [Na\(^{+}\)]pip (with [K\(^{+}\])o fixed at 18 mM), three-way ANOVA (n = 826) indicated significant group (P < 0.0001), voltage (P < 0.0001) and [Na\(^{+}\)]pip (P < 0.0001) main effects. In addition, the group × voltage (P = 0.0005) and group × [Na\(^{+}\)]pip (P < 0.0001) interaction effects were highly significant, indicating that the inherent differences in Ip between GFP and PLM myocytes were amplified at more positive voltages and higher [Na\(^{+}\)]pip. There were no other significant interaction effects.

The voltage dependence of K\(_{0.5,Na^{+}}\) and I\(_{\text{max}}\) of GFP and PLM myocytes is shown in Fig. 3, B and C, respectively. There were no apparent differences in K\(_{0.5,Na^{+}}\) between GFP and PLM myocytes although I\(_{\text{max}}\) was clearly suppressed with PLM overexpression. Across the physiological voltage range from −70 to +60 mV, fitted values of n\(_{h}\) varied between 2.74 to 3.77 (mean 3.27 ± 0.09) for GFP myocytes and between 2.66 to 4.13 (mean 3.22 ± 0.11) for PLM myocytes. The coefficient of determination (r\(^2\)) was ≥0.9632 for GFP myocytes and ≥0.9596 for PLM myocytes across the voltage range examined.

The relationship between Ip and [K\(^{+}\])o (with [Na\(^{+}\)]pip saturating at 80 mM) measured at −10 mV is shown in Fig. 4A. Fitted values for K\(_{0.5,K^{+}}\) are 2.69 ± 0.32 and 3.18 ± 0.36 mM, for I\(_{\text{max}}\) are 1.17 ± 0.08 and 0.73 ± 0.06 pA/pF, and for n\(_{h}\) are 1.96 ± 0.23 and 1.95 ± 0.77 for GFP and PLM myocytes, respectively. The coefficient of determination (r\(^2\)) is 0.9774 and 0.9686 for GFP and PLM myocytes, respectively.

When Ip was analyzed as a function of group (GFP vs. PLM), voltage, and [K\(^{+}\])o, (with [Na\(^{+}\)]pip fixed at 80 mM), three-way ANOVA (n = 987) indicated significant group (P < 0.0001), voltage (P < 0.0001) and [K\(^{+}\])o (P < 0.0001) main effects. In addition, the group × [K\(^{+}\])o (P < 0.0001) interaction effects were highly significant, indicating that the inherent differences in Ip between GFP and PLM myocytes were amplified at higher [K\(^{+}\])o. There were no other significant interaction effects.

The voltage dependence of K\(_{0.5,K^{+}}\) and I\(_{\text{max}}\) of GFP and PLM myocytes are shown in Fig. 4, B and C, respectively. Similar to K\(_{0.5,Na^{+}}\) (Fig. 3B), we did not detect a large effect of PLM overexpression on K\(_{0.5,K^{+}}\). Again, PLM overexpression clearly depressed I\(_{\text{max}}\). From −70 to +60 mV, fitted values of n\(_{h}\) varied between 1.46 to 2.50 (mean 1.80 ± 0.07) for GFP myocytes and between 1.34 to 2.63 (mean 1.87 ± 0.12) for PLM myocytes. Across the voltage range examined, r\(^2\) was ≥0.9591 and ≥0.9466 for GFP and PLM myocytes, respectively.

Effects of PLM overexpression on Na\(^{+}\)-K\(^{+}\)-ATPase protein and messenger RNA levels in adult rat myocytes. We investigated whether PLM overexpression affected transcription of α-subunits of Na\(^{+}\)-K\(^{+}\)-ATPase by performing real-time RT-PCR studies. Real-time RT-PCR analysis demonstrated amplification of each of the primer sets specific to each of the Na\(^{+}\)-K\(^{+}\)-ATPase α\(_{1}\)- and α\(_{2}\)-isoforms and no amplification of the no-template control samples, and the heat dissociation...
curve confirmed amplification of only a single gene transcript for each primer set (data not shown). These results indicate the presence of gene transcripts of the Na\(^+/\)K\(^+\)-ATPase \(\alpha_1\)- and \(\alpha_2\)-isoforms in GFP and PLM myocytes. For \(\alpha_1\)-isoform of Na\(^+/\)K\(^+\)-ATPase, the range of relative messenger RNA levels for GFP and PLM myocytes is 0.78–1.27 and 0.89–0.97, respectively. For \(\alpha_2\)-isoform of Na\(^+/\)K\(^+\)-ATPase, the range of relative messenger RNA levels for GFP and PLM myocytes is 0.87–1.15 and 0.81–1.01, respectively. Overexpression of PLM in adult rat myocytes did not result in detectable changes in messenger RNA levels of either \(\alpha_1\) or \(\alpha_2\)-isoforms of Na\(^+/\)K\(^+\)-ATPase (Fig. 5A).

To confirm our RT-PCR results, we next measured \(\alpha_1\)- and \(\alpha_2\)-subunits of Na\(^+/\)K\(^+\)-ATPase, using \(\alpha_1\)- and \(\alpha_2\)-specific antibodies. We did not detect significant differences in either \(\alpha_1\)- (GFP: 7.8 ± 1.5, PLM: 11.8 ± 1.9 arbitrary units; \(P = 0.17\)) or \(\alpha_2\)-subunits (GFP: 7.5 ± 1.1, PLM: 7.1 ± 1.0 arbitrary units; \(P = 0.79\)) of Na\(^+/\)K\(^+\)-ATPase (Fig. 5B). Equal protein loading in the lanes was verified by similar calsequestrin levels (data not shown).

Association of PLM with Na\(^+/\)K\(^+\)-ATPase in adult rat myocytes. Inhibition of Ip by PLM overexpression suggests that PLM may directly interact with Na\(^+/\)K\(^+\)-ATPase. We next sought to examine possible association between PLM and Na\(^+/\)K\(^+\)-ATPase. Figure 6 demonstrates that C2 Ab, but not preimmune serum (obtained from the same rabbit from which C2 Ab was raised), when covalently linked to agarose support beads, was able to immunoaffinity purify both PLM and \(\alpha_1\)-subunits of Na\(^+/\)K\(^+\)-ATPase present in native adult rat myocyte lysates.

PLM overexpression did not affect relative level of PLM phosphorylation at serine\(^{68}\). Using C68P Ab, an antibody that is specific for PLM phosphorylated at serine\(^{68}\) (27, 31), we demonstrated that, in rat cardiac myocytes, phosphorylated

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**Fig. 4.** Dependence of Ip on \([K^+]_o\), and voltage dependence of \(K_{50,K^+}\) and \(I_{\text{max}}\) in GFP and PLM myocytes. Ip at 80 mM \([Na^+]_o\) and 30°C was measured in GFP and PLM myocytes. \([K^+]_o\) was varied between 1 to 18 mM. A: Ip (measured at −10 mV) as a function of \([K^+]_o\), in control GFP (○) and PLM (●) myocytes. Each point represents mean ± SE of 4–10 myocytes. Mean Ip values are fitted to the Hill equation, and the fitted curves are shown for GFP (broken line) and PLM (solid line) myocytes, from which \(m_b\), \(l_{\text{max}}\), and \(K_{0.5,K^+}\) are obtained. Fitted values of \(K_{0.5,K^+}\) (B) and \(I_{\text{max}}\) (C) derived from data relating mean Ip and \([K^+]_o\), from −70 to +60 mV are shown for GFP (○) and PLM (●) myocytes.

**Fig. 5.** Effects of PLM overexpression on mRNA and protein levels of \(\alpha\)-subunits of Na\(^+/\)K\(^+\)-ATPase. A: cDNA was synthesized from total RNA isolated from GFP and PLM myocytes and used in real-time RT-PCR studies with appropriate primers for \(\alpha_1\)- and \(\alpha_2\)-isoforms of Na\(^+/\)K\(^+\)-ATPase (METHODS). Messenger RNA levels (normalized to 18S) relative to control GFP myocytes for \(\alpha_1\)- and \(\alpha_2\)-subunits of Na\(^+/\)K\(^+\)-ATPase are shown. Messenger RNA levels were determined twice with similar results. B: antibodies specific for \(\alpha_1\)- or \(\alpha_2\)-subunits of Na\(^+/\)K\(^+\)-ATPase were used in immunoblotting of GFP and PLM myocytes lysates. Protein band signal intensities are given in RESULTS.

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**Fig. 216 PHOSPHOLEMMAN REGULATES CARDIAC Na\(^+/\)K\(^+\)-ATPASE**

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**J Appl Physiol • VOL 100 • JANUARY 2006 • www.jap.org**
PLM detected by C68P Ab increased with phorbol 12-myristate-13-acetate or isoproterenol treatment, whereas the unphosphorylated PLM form detected by C2Ab showed the corresponding decrease (Fig. 7A). Using a 3rd PLM antibody that was engineered to recognize the NH2 terminus of PLM (and therefore recognizes both phosphorylated and unphosphorylated forms of PLM), we showed that protein loading was similar in all three lanes (Fig. 7A). Assuming that either PKA or PKC stimulation resulted in 100% phosphorylation at serine68 under our experimental conditions, we estimated that 41 ± 9% of serine68 in PLM was phosphorylated in native rat cardiac myocytes under basal conditions (n = 4). To further demonstrate that C2 Ab and C68P Ab can detect changes in unphosphorylated and phosphorylated forms of PLM, respectively, we treated lysates prepared from PMA-stimulated myocytes with bacterial alkaline phosphatase. As shown in Fig. 7B, alkaline phosphatase treatment of PMA-treated myocyte lysates resulted in decrease in signal intensity detected by C68P Ab but increase in signal intensity detected by C2 Ab.

In PLM overexpressed myocytes, the unphosphorylated PLM (as detected by C2 Ab) was 108.6 ± 3.0 arbitrary units compared with 6.5 ± 2.1 arbitrary units in GFP myocytes (n = 6; P < 0.0001) (Fig. 7C). The increase in unphosphorylated PLM in PLM-overexpressed myocytes was ~16.7-fold compared with GFP myocytes. By comparison, PLM phosphorylated at serine68 (as detected by C68P Ab) was 3.7 ± 0.5 arbitrary units in GFP and 41.8 ± 3.9 arbitrary units in PLM myocytes (n = 6; P < 0.0001) (Fig. 7C). The mean increase in phosphorylated PLM in PLM myocytes was ~11.3-fold compared with GFP myocytes. Given the similar fold increase in both unphosphorylated and phosphorylated PLM in PLM overexpressed myocytes, PLM overexpression did not grossly affect the relative degree of serine68 phosphorylation in rat myocytes.

**DISCUSSION**

PLM (FXYD1) belongs to the FXYD gene family of small ion transport regulators (36). Interestingly, the γ-subunit of the Na+-K+-ATPase (FXYD2) is also a member of the FXYD family and is only present in membranes from kidney tubules (39). In the actively transporting nephron, the γ-subunit regulates Na+-K+-ATPase activity by stabilizing the E1 conformation of the enzyme (26, 38, 39). A 15-kDa homolog of

![Fig. 7. Serine68 phosphorylation in PLM. A: freshly isolated rat myocytes were treated with phorbol 12-myristate-13-acetate (PMA, 0.5 μM), isoproterenol (Iso, 0.5 μM), or dimethylsulfoxide (Control, 0.55 μg/ml) at 37°C for 10 min. Myocyte homogenates were prepared and immunoblotting performed with C2 Ab, C68P Ab, and a polyclonal antibody that recognizes the NH2 terminus of PLM (to control for PLM loading). This experiment was repeated 3 times with similar results. B: myocytes treated with either dimethylsulfoxide (Control) or PMA were scraped into 500 l of lysis buffer without phosphatase inhibitor cocktail, Na+ vanadate, and NaF. A portion of myocyte lysate (100 μl) was treated with bacterial alkaline phosphatase (AKLP, 21.6 μg) at 37°C for 30 min. Phosphatase inhibitor cocktail (4 μl) was added to the remaining 400 μl of myocyte lysate. Phosphorylated and unphosphorylated PLM in the myocyte lysate were analyzed with C2 Ab and C68P Ab, respectively. C: immunoblotting was performed on cell lysates prepared from GFP and PLM myocytes (n = 6 each) with either C2 Ab or C68P Ab. Protein signal band intensities are given in RESULTS.](http://www.jap.org/content/100/1/217/F1)
PLM isolated from shark rectal glands, PLMS, also associated with the α-subunits of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (18). Phosphorylation of PLMS by PKA or PKC resulted in partial dissociation of PLMS from the α-subunit of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase, with subsequent activation of the enzyme (18). In addition, recent studies indicate that CHIP (channel inducing factor; FXYD4) (2) and FXYD7 (3) were also regulators of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase. Thus, within the FXYD family, at least four members (γ-subunit of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase, CHIP, FXYD7, and PLMS) other than PLM (6) associated with the α-subunit of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase or regulated its activity.

The first major finding of the present study is that we demonstrated that PLM protein levels were significantly elevated in postinfarction rat hearts (Fig. 1). Our findings are in agreement with microarray data indicating that messenger RNA levels of PLM were increased postinfarction (29).

In our experiments, Ip reached plateau amplitude at ~0 mV (Fig. 2D); a characteristic of the pump current that was first described by Gadsby et al. (12) and confirmed by others (34). The fitted amplitude of $I_{\text{max}}$ (1.39 ± 0.02 pA/pF) in control rat myocytes measured at +60 mV and 80 mM [Na\textsuperscript{+}] pip (Fig. 4C) was similar to that previously reported by other investigators (1.8–2.0 pA/pF at +50 mV; 50 mM [Na\textsuperscript{+}] pip and 5.4 mM [K\textsuperscript{+}]; Ref. 34). In addition, values for $K_{0.5}$,Na1 (8.9–12.6 mM; Fig. 3B) and $K_{0.5}$,Na2 (2.5–3.1 mM; Fig. 4B) were similar to those reported for $K_{0.5}$,Na (7.8–40 mM) and $K_{0.5}$,K (0.9–3.5 mM) for rat ventricular myocytes (for review, see Ref. 13). These observations indicate that the ouabain-sensitive current measured under our experimental conditions was indeed the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase pump current.

The second major finding of the present study is that overexpression of PLM inhibited Ip by as much as 27–40% (Figs. 2–4). Inhibition of Ip by PLM was not due to sequestration of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase by the overexpressed PLM molecules in subcellular organelles. This is because we have previously demonstrated by indirect immunofluorescence that the overexpressed PLM was correctly targeted to sarcotema, T tubules, and intercalated discs, with little to no PLM signal detected in the cytosol (32, 42). Inhibition of Ip by overexpressed PLM was also not due to decreased expression of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase, as demonstrated by no decreases in messenger RNA and protein levels of α1- and α2-subunits of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (Fig. 5). In contrast to the results derived from co-overexpression of PLM and α1,β1 Na\textsuperscript{+}-K\textsuperscript{+}-ATPase isoenzymes in Xenopus oocytes (6), in its native environment of cardiac sarcotema, PLM overexpression decreased maximum Ip with no large effects on $K_{0.5}$,Na1 or $K_{0.5}$,K (Figs. 3 and 4). Our data agree well with a recent report that demonstrated that the $V_{\text{max}}$ of sarcolemmal Na\textsuperscript{+}-K\textsuperscript{+}-ATPase was increased threefold after acute cardiac ischemia, presumably because of less inhibition of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase by PLM whose phosphorylation status was enhanced by >300% (10). Our present results indicate that in addition to regulation of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger activity (1, 20, 32, 42), another potential function of PLM in cardiac tissues is to modulate Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity.

In adult rat myocardium, both α1- and α2-subunits of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase are expressed in the sarcotema and T tubules of cardiac myocytes, with the α1-subunit perhaps more abundant in the T tubules (19, 35). Expression of α2-subunit of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in adult rat hearts has been reported by some (28, 30) but not by other investigators (19, 35). In addition, ~75% of the Na\textsuperscript{+} pump αβ-heterodimers in the adult rat heart consisted of the fairly ubiquitous but relatively ouabain-resistant α1-isoforms (19, 35). In our experiments, we used ouabain at 1 mM, which is much higher than the $K_{i}$ for the α1-isoform and thus should result in inhibition of both the α1- and α2-isoforms of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase. Our experimental conditions, therefore, could not differentiate whether PLM modulated α1- and/or α2-isoforms of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase to account for the observed inhibition of Ip with PLM overexpression.

The third finding is that PLM coassociates with the α-subunits of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (Fig. 6), in agreement with the observations by other investigators (6, 10, 31). The α2-antibody that we used to detect the α-subunits of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase does not distinguish between α1- and α2-isoforms of the enzyme. Association of the α-subunits of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase with PLM constitutes another line of evidence that PLM regulates Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity. Recent studies using immunoprecipitation and indirect immunofluorescence localization techniques indicated that PLM associated with α1 but not the α2-subunit of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in guinea pig myocytes (10, 31). Taken together, it is likely that PLM regulates the activity of α1-subunit of cardiac Na\textsuperscript{+}-K\textsuperscript{+}-ATPase.

A fourth major finding is that by assuming that either PKA or PKC stimulation resulted in 100% phosphorylation of serine68 and that C68P Ab and C2 Ab detected only the phosphorylated and unphosphorylated forms of PLM, respectively (27, 31), we estimated that ~41% of serine68 in PLM was phosphorylated in rat myocytes under the bas al state. This estimate agrees reasonably well with the ~46% phosphorylation of serine68 of PLM in resting rat myocytes, on the basis of comparing the effects of wild-type PLM and its serine68 mutants on Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange current (32). Using C68P Ab, Silverman et al. (31) showed that forskolin treatment of guinea pig myocytes resulted in approximately fourfold increase in serine68 phosphorylation. Assuming forskolin induced 100% phosphorylation of serine68, basal serine68 phosphorylation can be estimated to be ~25% in guinea pig myocytes.

On the basis of analogy of phospholamban inhibition of SERCA2 and experimental observations on the effects of PLMS on shark Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (18), the current working model is that the Na\textsuperscript{+} pump is inhibited by unphosphorylated PLM. On phosphorylation of PLM, inhibition of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase is relieved. This hypothesis has been given indirect support by the observation that the $V_{\text{max}}$ of sarcolemmal Na\textsuperscript{+}-K\textsuperscript{+}-ATPase was increased threefold after acute cardiac ischemia, in association with increased PLM phosphorylation by >300% (10). It is at present not clear whether dissociation of the phosphorylated PLM from Na\textsuperscript{+}-K\textsuperscript{+}-ATPase is required to relieve its inhibition on the Na\textsuperscript{+} pump (10, 18, 31). An important finding of the present study is that PLM overexpression did not grossly distort the relative level of serine68 phosphorylation. This indicates that suppression of Ip in PLM overexpressed myocytes was due to increased unphosphorylated PLM forms.

The characteristic phenotype observed in adult rat myocytes 3–9 wk post-MI is that when compared with sham myocytes, at 0.6 mM [Ca\textsuperscript{2+}], both contraction and [Ca\textsuperscript{2+}], transient amplitudes were higher. At 5 mM [Ca\textsuperscript{2+}], both contraction and [Ca\textsuperscript{2+}], transient amplitudes were lower in MI than sham myocytes (5, 41, 43). This reduced dynamic range of contraction and [Ca\textsuperscript{2+}], transient amplitudes in response to increasing
[Ca\(^{2+}\)]_o was also observed in rat myocytes in which Na\(^+/\text{Ca}\(^{2+}\)) exchange was downregulated (37). In post-MI rat myocytes, both Na\(^+/\text{Ca}\(^{2+}\)) exchanger expression (30) and its activity (45) were depressed. In addition, contractile and [Ca\(^{2+}\)] transient abnormalities in post-MI rat myocytes were rescued by overexpressing the Na\(^+/\text{Ca}\(^{2+}\)) exchanger (43). Taken together, these observations suggest that the contractile and [Ca\(^{2+}\)] homeostatic abnormalities in post-MI rat myocytes were in large part due to decreased Na\(^+/\text{Ca}\(^{2+}\)) exchange activity. In this context, it is interesting to note that PLM, in addition to its effects on Na\(^+/\text{K}\(^{+}\))-ATPase, also inhibits the cardiac Na\(^+/\text{Ca}\(^{2+}\)) exchanger (1, 32, 42). In addition, overexpression of PLM in normal rat myocytes resulted in reduced dynamic range of contraction and [Ca\(^{2+}\)], transient amplitudes in response to increasing [Ca\(^{2+}\)]_o (33), similar to the contractile phenotype observed in post-MI rat myocytes (5, 41, 43). Thus, in post-MI rat myocytes, contractile and [Ca\(^{2+}\)] transient abnormalities were largely due to decreased expression of Na\(^+/\text{Ca}\(^{2+}\)) exchanger (30) and PLM overexpression with its attendant inhibitory effects of Na\(^+/\text{Ca}\(^{2+}\)) exchange activity (1, 32, 42). On the other hand, inhibition of Na\(^+/\text{K}\(^{+}\))-ATPase by PLM overexpression (Figs. 2–4) or ouabain (14) would be expected to elevate [Na\(^{+}\)], resulting in changes in thermodynamic driving force for Na\(^+/\text{Ca}\(^{2+}\)) exchange. This would reduce Ca\(^{2+}\) extrusion by forward Na\(^+/\text{Ca}\(^{2+}\)) exchange but increase Ca\(^{2+}\) influx by reverse Na\(^+/\text{Ca}\(^{2+}\)) exchange. The net result would be increased contraction and [Ca\(^{2+}\)], transient amplitudes in PLM overexpressed or ouabain-treated myocytes under both low and high [Ca\(^{2+}\)], conditions, a prediction not consistent with our observation in PLM overexpressed or post-MI myocytes. Viewed in this simplistic context, decreased Na\(^+/\text{K}\(^{+}\))-ATPase activity is unlikely to be a major factor to account for altered contractility in post-MI myocytes. Na\(^+/\text{K}\(^{+}\))-ATPase, however, is intimately involved with cell volume regulation (17) and affects automaticity, resting, and action potentials (13). Inhibition of Na\(^+/\text{K}\(^{+}\))-ATPase by PLM, therefore, may have profound consequences during cardiac ischemia when disordered cell volume regulation and electrical instability are present.

There are limitations to the present study. The first is that we did not address the stoichiometry of interaction between PLM and Na\(^+/\text{K}\(^{+}\))-ATPase because the amounts of \(\alpha_1\)-subunits of Na\(^+/\text{K}\(^{+}\))-ATPase, PLM, and NCX1 present in a cardiac cell are at present unknown. In addition, there may be as yet unknown partners that PLM interacts with. Furthermore, PLM may form dimers in the sarcolemma (Cheung JY, unpublished observations). Therefore, precise quantification of the stoichiometry of interaction between PLM and Na\(^+/\text{K}\(^{+}\))-ATPase in the cardiac cell is exceedingly complex and beyond the scope of the present study. Nevertheless, qualitative mechanisms by which the overexpressed unphosphorylated PLM decreases the \(V_{\text{max}}\) of Na\(^+/\text{K}\(^{+}\))-ATPase without affecting the \(\alpha\)-subunit content can be speculated. The first is that there exists a pool of Na\(^{+}\) pump that is not associated with PLM in normal cardiac myocytes and that the overexpressed unphosphorylated PLM interacts with it. Another possibility is that the overexpressed unphosphorylated PLM successfully displaces the phosphorylated PLM from the Na\(^{+}\) pump. The second limitation is that the level of PLM overexpression by adenovirus infection varied between 1.4-fold (33), 2.6- to 3.5-fold (42), and 11- to 17-fold (present study), depending on the batch of adenovirus constructs and the multiplicity of infection (2 to 5) used. The range of PLM increase with adenovirus-mediated gene delivery observed in our previous (33, 42) and present study, however, is within the range of PLM increase observed in postinfarction myocytes.

In summary, we have demonstrated elevated PLM protein levels in postinfarction rat myocytes. PLM overexpression in normal rat myocytes resulted in depressed Na\(^+/\text{K}\(^{+}\))-ATPase pump current that was primarily due to decreases in \(V_{\text{max}}\) with no significant changes in apparent affinities for Na\(^{+}\) and K\(^{+}\); changes similar to those observed in postinfarction rat myocytes. PLM overexpression did not affect messenger RNA and protein levels of \(\alpha_1\)- or \(\alpha_2\)-subunits of Na\(^+/\text{K}\(^{+}\))-ATPase, nor did it affect the relative phosphorylation level of serine68 of PLM. Immunoaffinity purification experiments demonstrated association of \(\alpha\)-subunits of Na\(^+/\text{K}\(^{+}\))-ATPase with PLM. We conclude that PLM regulates Na\(^+/\text{K}\(^{+}\))-ATPase activity in the heart. We speculate that, in addition to reduced expression of Na\(^+/\text{K}\(^{+}\))-ATPase (28, 30), overexpression of PLM may also account for depressed Na\(^+/\text{K}\(^{+}\))-ATPase activities observed in postinfarction rat myocytes.

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