Overexpression of phospholemman alters contractility and 
$[\text{Ca}^{2+}]_i$ transients in adult rat myocytes

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Overexpression of phospholemman alters contractility and 

Previous studies showed increased phospholemman (PLM) mRNA after myocardial infarction (MI) in rats (Sehl PD, Tai JTN, Hillan KJ, Brown LA, Goddard A, Yang R, Jin H, and Lowe DG. Circulation 101: 1990–1999, 2000). We tested the hypothesis that, in normal adult rat cardiac myocytes, PLM overexpression alters contractile function and cytosolic $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_i$) homeostasis in a manner similar to that observed in post-MI myocytes. Compared with myocytes infected by control adenovirus expressing green fluorescent protein (GFP) alone, Western blots indicated a 41% increase in PLM expression after 72 h ($P<0.001$) but no changes in Na$^+$/Ca$^{2+}$ exchanger, SERCA2, and calsequestrin levels in myocytes infected by adenovirus expressing GFP and PLM. At 5 mM extracellular $[\text{Ca}^{2+}]_o$, maximal contraction amplitudes in PLM-overexpressed myocytes were 24% ($P<0.005$) and $[\text{Ca}^{2+}]_i$, transient amplitudes were 18% ($P<0.05$) lower than control myocytes. At 0.6 mM $[\text{Ca}^{2+}]_o$, however, contraction and $[\text{Ca}^{2+}]_i$, transient amplitudes were significantly ($P<0.05$) higher in PLM-overexpressed than control myocytes (18% and 42%, respectively); at 1.8 mM $[\text{Ca}^{2+}]_o$, the differences in contraction and $[\text{Ca}^{2+}]_i$, transient amplitudes were narrowed. This pattern of contractile and $[\text{Ca}^{2+}]_i$ homeostasis in a manner similar to that observed in post-MI rat myocytes. We suggest that PLM overexpression observed in post-MI rat myocytes may partly account for contractile abnormalities by perturbing $\text{Ca}^{2+}$ fluxes during excitation–contraction.

primary adult cardiac myocyte culture; fura 2; edge detection; sarco(endo)plasm reticulum calcium adenosinetriphosphatase

PHOSPHOLEMMAN (PLM), a 72-amino acid integral membrane phosphoprotein with a single transmembrane domain, is a major sarcolemmal substrate for protein kinases A and C in heart and skeletal muscle (14, 18, 20). Its physiological function is largely unknown. Early work based on overexpression of PLM in Xenopus oocytes suggested that PLM was a hyperpolarization-activated anion-selective channel (16). More recent studies, however, indicated that PLM interacts with endogenous oocyte anion channels because expression of nonchannel hydrophobic peptides induced similar currents in Xenopus oocytes (22, 25). When reconstituted in lipid bilayers, PLM formed a channel that was highly selective for taurine (2). In addition, it was recently recognized that PLM belongs to the FYXD family of small ion transport regulators (23). Thus current evidence suggests that PLM 1) can be a channel, a channel subunit, or an ion transport regulator; 2) is a major substrate for phosphorylation; and 3) very likely interacts with other proteins.

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 1 of only 19 genes to increase after myocardial infarction (MI) (21). Specifically, when compared with sham-operated rat ventricles, PLM expression was increased twofold as early as 3 days after MI and remained elevated for at least 2 wk after MI. The effects of increased PLM on myocyte function are unknown. The present study was undertaken to evaluate whether PLM overexpression in normal adult rat cardiac myocytes alters contractile function and cytosolic $[\text{Ca}^{2+}]_i$ homeostasis in a manner similar to that observed in post-MI myocytes (3, 30, 31).

METHODS

PLM polyclonal antibody. Polyclonal antibody was raised against a 16-amino acid peptide fragment of the COOH terminus of PLM (NH$_2$-CGTFRSSIRRLSTRRR-COOH). To verify that the affinity-purified rabbit polyclonal PLM antibodies could indeed recognize PLM, we performed the following study. The coding sequence of canine heart PLM in pAlter-1 (a generous gift from Dr. J. R. Moorman, University of Virginia, Charlottesville, VA) was amplified by PCR with the following set of primers: 5'-GAA TTC CAT ATG GAA GCG CCA CAG GAA CAC-3' and 5'-AAG CTT CCT TCC GAG CTA CTA CCG CCT GCG GMT-3'. The PCR product (246 bp) contained EcoRI and NdeI restriction sites at the 5' end and XhoI and HindIII restriction sites at the 3' end of the PLM open reading frame. The PCR product was cloned into the pAlter-1 vector.

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sequence. After digestion with NdeI and XhoI, the PLM sequence was inserted into pET-19b, using the same NdeI and XhoI restriction sites on the cloning vector (Novagen, Madison, WI). The pET-19b vector contained an NH2-terminus His tag sequence followed by an enterokinase site upstream of the NdeI and XhoI cloning sites. The cloned pET-19b-PLM plasmid was used to transform BL21(DE3)pLysS cells, and PLM expression was induced by isopropyl-β-D-thiogalactopyranoside. Bacterial lysate containing recombinant His-tagged PLM was mixed with Ni-NTA agarose slurry (Qiagen, Valencia, CA) and applied to a column, and His-tagged PLM was eluted and then dialyzed overnight against 2 M urea in phosphate-buffered saline. His-tagged PLM (1 μg/lane) in SDS sample buffer was subjected to 12% PAGE and transferred onto Immun-Blot polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). His-tagged PLM was detected with murine monoclonal anti-polyhistidine antibody (1:5,000) against the COOH terminus of PLM to the blot resulted in detection of both enterokinase-treated PLM (lane C) and His-tagged PLM (lane D).

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[Ca^{2+}], transient measurements. Myocytes were exposed to 0.67 μM of fura 2-AM for 15 min at 37°C. Fura 2-loaded myocytes mounted in a Dvorak-Stotler chamber situated in a temperature-controlled stage (37°C) of a Zeiss IM35 inverted microscope were field stimulated to contract at 1 Hz between platinum wire electrodes (29, 31, 32). [Ca^{2+}], was varied between 0.6 and 5.0 mM. [Ca^{2+}], transient measurements, calibration of fura 2 fluorescence signals, and [Ca^{2+}], transient analyses were performed as previously described (29, 31, 32).

PLM, Na^{+}/Ca^{2+} exchanger, sarc(o)endoplasmic reticulum Ca^{2+}-ATPase, and casquestrin immunoblotting. Cultured myocytes were harvested for immunoblotting on day 3. Cultured myocytes in a four-well tray were rinsed three times with ice-cold phosphate-buffered saline. They were then scraped into 1 ml of ice-cold lysis buffer containing (in mM) 50 Tris (pH 8.0), 150 NaCl, 1 Na^{+} orthovanadate, 1 phenylmethylsulfonyl fluoride, 100 NaF, 1 EDTA, and 1 EGTA, with 0.5% NP-40, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The cell lysate was snap-frozen in dry ice-ethanol and stored at −80°C.

Myocyte lysates in SDS sample buffer (containing either 10 mM N-ethylmaleimide (for Na^{+}/Ca^{2+} exchanger) or 5% 2-mercaptoethanol (for PLM and sarc(o)endoplasmic reticulum Ca^{2+}-ATPase (SERCA2))) were subjected to 7% (SERCA2, Na^{+}/Ca^{2+} exchanger, and casquestrin) or 12% (PLM) PAGE (29, 31, 32). The fractionated proteins were transferred onto Immob-Blot PVDF membranes. Rabbit polyclonal antibodies against PLM COOH terminus (1:500) were used to detect PLM, with donkey anti-rabbit IgG (1:5,000; Amersham) as the secondary antibody. SERCA2 was detected with a monoclonal antibody (1:1,000, MAN-919; Affinity Bioreagents, Golden, CO), and sheep anti-mouse antibody (1:2,000; Amersham) was used as the secondary antibody. For casquestrin immunoblotting, membranes stripped of Na^{+}/Ca^{2+} exchanger or SERCA2 were sequentially exposed to rabbit anti-calsequestrin antibody (1:2,500; Swant) and donkey anti-rabbit IgG (1:5,000; Amersham) as the secondary antibody. The secondary antibody was used as the secondary antibody. For casquestrin immunoblotting, membranes stripped of Na^{+}/Ca^{2+} exchanger or SERCA2 were sequentially exposed to rabbit anti-calsequestrin antibody (1:2,500; Swant) and donkey anti-rabbit IgG (1:5,000; Amersham). Immunoreactive proteins were detected with the enhanced chemiluminescence-Western blotting system. Protein band signal intensities were quantitated by scanning autoradiograms of the blots with a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Statistics. All results are expressed as means ± SE. In experiments in which maximal contraction amplitudes were measured as a function of experimental group (−Adv-GFP vs. +Adv-GFP), [Ca^{2+}], and days in culture, three-way ANOVA was performed to determine significance of difference. A linear model-fitted standard least squares (JMP version 4; SAS Institutes, Cary, NC) was used. For analysis of a parameter (e.g., systolic [Ca^{2+}], maximal contraction amplitude) as a function of group (GFP vs. PLM) and [Ca^{2+}], two-way ANOVA was used to determine statistical significance. Paired Student’s t-test was used to compare protein abundance between GFP and PLM myocytes. In all analyses, P < 0.05 was taken to be statistically significant.

RESULTS

Effects of continual pacing and adenoviral infection on cultured myocyte contractility. We (27) and others (8) have demonstrated that adult rat cardiac myocytes cultured under quiescent conditions for as little as 6–18 h exhibited deterioration in cell shortening compared with freshly isolated myocytes. Maximal contraction amplitudes (1 Hz) of adult myocytes placed under continual pacing culture conditions were examined on days 0 (freshly isolated), 1, 2, and 3 and are shown in Fig. 2. Inspection of Fig. 2 suggests that for myocytes stimulated at 0.6 and 1.8 mM [Ca^{2+}], there were no differences in maximal contraction amplitudes on all 4 days. For myocytes stimulated at 5 mM [Ca^{2+}], maximal contraction amplitude declined modestly (from 17.7 ± 1.74 to 12.1 ± 1.576 ± 3.51% resting cell length) after 3 days of culture. This conclusion is supported by two-way ANOVA ([Ca^{2+}], day in culture), which indicated significant [Ca^{2+}], (P < 0.0001) and day in culture (P = 0.0174) effects. When data obtained at 5 mM [Ca^{2+}], were removed from analysis, two-way ANOVA indicated significant [Ca^{2+}], (P < 0.0001) effect, but time in culture had no effect on myocyte contraction amplitudes.

Infection with recombinant, replication-defective adenovirus provided an useful method to introduce exogenous genes into adult cardiac myocytes (10, 12, 32). Compared with uninfected myocytes, Adv-GFP-infected myocytes at 6 (day 0), 24, 48, and 72 h of continual pacing in culture had similar contraction amplitudes, regardless of whether cell shortening was measured at 0.6, 1.8, or 5.0 mM [Ca^{2+}], (Fig. 2). Indeed, three-way ANOVA (group, [Ca^{2+}], and day) indicated no significant (P = 0.0919) differences in maximal contraction amplitudes between noninfected and adenovirus-infected myocytes.

![Fig. 2. Effects of continuous pacing culture and adenovirus infection on adult rat cardiac myocyte contractility. Isolated myocytes infected with recombinant adenovirus that expressed green fluorescent protein (GFP; filled symbols) were cultured at extracellular Ca^{2+} concentration ([Ca^{2+}],) of 1.8 mM for 72 h under continuous electrical stimulation (1 Hz) conditions (32). Myocytes not infected with adenovirus (open symbols) served as controls. For contraction studies, myocytes taken out of culture on different days were paced (1 Hz) to contract at 37°C and [Ca^{2+}], of 0.6 (circles), 1.8 (diamonds), and 5.0 (squares) mM. Each point represents the mean ± SE of 14–38 observations. Error bars are not shown if they fall within the boundaries of the symbol. Statistical analyses are given in RESULTS.](http://ajpheart.physiology.org/content/283/4/11001)
Effects of Adv-GFP-PLM infection on PLM abundance. Seventy-two hours after infection with either Adv-GFP or Adv-GFP-PLM, cardiac myocyte lysates were collected for analysis for PLM abundance by immunoblotting. Under reducing conditions, native cardiac PLM migrated on SDS-PAGE with an apparent molecular mass of 15 – 16 kDa (Fig. 3; Ref. 2). Compared with GFP myocytes, PLM myocytes had a significant 1.4-fold increase in PLM (Fig. 3; Table 1). By contrast, there were no significant differences in Na\(^{+}\)/H\(^{+}\)/Ca\(^{2+}\)/H\(^{+}\) exchanger, SERCA2, or calsequestrin protein amounts between GFP and PLM myocytes (Fig. 3; Table 1).

Effects of PLM overexpression on contractile function. Preliminary studies indicated no significant differences in contraction amplitudes between GFP and PLM myocytes when they were examined 48 h after adenoviral infection (data not shown). Therefore, all contraction and [Ca\(^{2+}\)]\(_{i}\) transient studies were performed after 72 h of adenoviral infection.

At 0.6 mM [Ca\(^{2+}\)]\(_{o}\), PLM myocytes shortened more than GFP myocytes (Fig. 4, A and B; Table 2). By contrast, at 1.8 and 5.0 mM [Ca\(^{2+}\)]\(_{o}\), PLM myocytes shortened less than GFP myocytes (Fig. 4, C–F; Table 2). These conclusions are supported by highly significant group ([Ca\(^{2+}\)]\(_{o}\), [Ca\(^{2+}\)]\(_{o}\) interaction effects. The significant group × [Ca\(^{2+}\)]\(_{o}\) interaction effect indicates that the magnitude and/or direction of the effects of [Ca\(^{2+}\)]\(_{o}\) on cell shortening was different across experimental groups (GFP vs. PLM).

As a group, maximal shortening velocities were lower in PLM myocytes (Table 2; significant group effect, \(P < 0.006\)) compared with GFP myocytes. Inspection of data in Table 2 suggests that maximal shortening velocities were not different at 0.6 mM [Ca\(^{2+}\)]\(_{o}\) but were significantly lower in PLM myocytes stimulated at 1.8 and 5.0 mM [Ca\(^{2+}\)]\(_{o}\) (significant

**Table 1. Effects of PLM overexpression on levels of selected proteins**

<table>
<thead>
<tr>
<th></th>
<th>GFP</th>
<th>PLM</th>
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<tbody>
<tr>
<td>PLM</td>
<td>349 ± 80 (5)</td>
<td>492 ± 68 (5)*</td>
</tr>
<tr>
<td>NCX1</td>
<td>1,307 ± 155</td>
<td>1,388 ± 115</td>
</tr>
<tr>
<td>SERCA2</td>
<td>1,071 ± 121</td>
<td>1,125 ± 214</td>
</tr>
<tr>
<td>Calsequestrin</td>
<td>1,814 ± 71</td>
<td>1,706 ± 67</td>
</tr>
</tbody>
</table>

Values (in arbitrary units) are means ± SE. Nos. in parentheses are nos. of hearts used in myocyte culture. GFP myocytes were infected with adenovirus expressing green fluorescent protein (GFP); PLM myocytes were infected with adenovirus expressing both GFP and canine heart phospholemman (PLM). *\(P < 0.001\), GFP vs. PLM, paired t-test. NCX1, Na\(^{+}\)/Ca\(^{2+}\) exchanger; SERCA2, sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase.

**Fig. 4.** PLM overexpression alters contractile function in adult rat myocytes. Isolated myocytes were infected with recombinant adenovirus expressing either GFP or both GFP and PLM and then cultured for 72 h under continuous electrical stimulation (1 Hz) conditions. For contraction studies, cultured myocytes were paced (1 Hz) to contract at 37°C and [Ca\(^{2+}\)]\(_{o}\) of 0.6, 1.8, or 5.0 mM. Shown are steady-state paced twitches from myocyte expressing either GFP (A, C, E) or PLM (B, D, F). Results are summarized in Table 2.
Table 2. Effects of PLM overexpression on myocyte shortening dynamics

<table>
<thead>
<tr>
<th>[Ca\textsuperscript{2+}]\textsubscript{o}</th>
<th>Maximal Contraction Amplitude, % RCL</th>
<th>Maximal Shortening Velocity, cell length/s</th>
<th>( t_{\text{1/2}} ), ms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GFP</td>
<td>PLM</td>
<td>GFP</td>
</tr>
<tr>
<td>0.6</td>
<td>4.97 ± 0.50 (21)</td>
<td>5.85 ± 0.44 (21)*</td>
<td>0.63 ± 0.06</td>
</tr>
<tr>
<td>1.8</td>
<td>12.03 ± 0.80 (21)</td>
<td>10.12 ± 0.46 (22)*</td>
<td>1.42 ± 0.10</td>
</tr>
<tr>
<td>5.0</td>
<td>16.14 ± 0.70 (24)</td>
<td>12.28 ± 0.48 (36)*</td>
<td>1.86 ± 0.10</td>
</tr>
</tbody>
</table>

Values are means ± SE. Nos. in parentheses are nos. of myocytes, without regard to the no. of cells contributed by each heart. [Ca\textsuperscript{2+}]\textsubscript{o}, extracellular Ca\textsuperscript{2+} concentration; RCL, resting cell length; \( t_{\text{1/2}} \), half-time of myocyte relaxation. Cell shortening dynamics were measured in myocytes after 72 h of adenovirus infection. *P < 0.005, PLM vs. GFP.

### DISCUSSION

Previous studies on PLM, a major substrate for protein kinases A and C in cardiac membranes (14, 18, 20), focused on its ion channel-like properties (16), channel regulatory roles (22), and cell volume regulatory func-

The table shows the effects of PLM overexpression on myocyte shortening dynamics. In both groups, raising [Ca\textsuperscript{2+}]\textsubscript{o} increased maximal shortening velocity (significant [Ca\textsuperscript{2+}]\textsubscript{o} effect, P < 0.0001). Half-times (\( t_{\text{1/2}} \)) of relaxation were not different between GFP and PLM myocytes (group effect, P = 0.29) across the three [Ca\textsuperscript{2+}]\textsubscript{o} concentrations examined (group × [Ca\textsuperscript{2+}]\textsubscript{o} interaction effect, P = 0.14).

**Effects of PLM overexpression on [Ca\textsuperscript{2+}]\textsubscript{i} transients.** Resting [Ca\textsuperscript{2+}]\textsubscript{i} values in quiescent myocytes before stimulation were not different between GFP and PLM myocytes (group effect, P = 0.64) across the range of [Ca\textsuperscript{2+}]\textsubscript{o} examined (group × [Ca\textsuperscript{2+}]\textsubscript{o} effect, P = 0.57; Table 3). Similarly, end-diastolic [Ca\textsuperscript{2+}]\textsubscript{i} levels in myocytes paced at 1 Hz were not different (P = 0.80) between the two groups (Table 3). Varying [Ca\textsuperscript{2+}]\textsubscript{o} had no effect on either resting [Ca\textsuperscript{2+}]\textsubscript{i} ([Ca\textsuperscript{2+}]\textsubscript{o} effect, P = 0.85) or diastolic [Ca\textsuperscript{2+}]\textsubscript{i} ([Ca\textsuperscript{2+}]\textsubscript{o} effect, P = 0.58) in both GFP and PLM myocytes (Table 3).

With respect to systolic [Ca\textsuperscript{2+}]\textsubscript{i}, at low (0.6 mM) [Ca\textsuperscript{2+}]\textsubscript{o}, measured values for PLM myocytes were higher than those found for GFP myocytes (Fig. 5, A and B; Table 3). At 1.8 and 5.0 mM [Ca\textsuperscript{2+}]\textsubscript{o}, however, systolic [Ca\textsuperscript{2+}]\textsubscript{i} was higher in GFP myocytes compared with PLM myocytes (Fig. 5, C–F; Table 3). In both groups, raising [Ca\textsuperscript{2+}]\textsubscript{o} increased systolic [Ca\textsuperscript{2+}]\textsubscript{i} (Table 3). Our interpretation is supported by two-way ANOVA: significant group (P = 0.04), [Ca\textsuperscript{2+}]\textsubscript{o} (P < 0.0001), and group × [Ca\textsuperscript{2+}]\textsubscript{o} interaction (P = 0.04) effects.

The magnitude of the [Ca\textsuperscript{2+}]\textsubscript{i} transient is reflected by the percent increase in fura 2 fluorescence intensity ratio, which has the advantage of being free from fluorescence signal calibration errors and uncertainties in intracellular fura 2 dissociation constant. Compared with control GFP myocytes, [Ca\textsuperscript{2+}]\textsubscript{i} transient amplitudes in PLM myocytes were higher at 0.6 mM [Ca\textsuperscript{2+}]\textsubscript{o} (Fig. 5, A and B; Table 3) but lower at 1.8 and 5.0 mM [Ca\textsuperscript{2+}]\textsubscript{o} (Fig. 5, C–F; Table 3). As expected, increasing [Ca\textsuperscript{2+}]\textsubscript{o} resulted in higher [Ca\textsuperscript{2+}]\textsubscript{i} transient amplitudes in both groups (Table 3). Two-way ANOVA indicated significant group (P = 0.03), [Ca\textsuperscript{2+}]\textsubscript{o} (P < 0.0001), and group × [Ca\textsuperscript{2+}]\textsubscript{o} interaction (P < 0.02) effects.

The \( t_{\text{1/2}} \) of [Ca\textsuperscript{2+}]\textsubscript{i} decline, an estimate of in situ sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} uptake activity (26, 31), was not different between GFP and PLM myocytes (Table 3; P = 0.65) at all three [Ca\textsuperscript{2+}]\textsubscript{o} values examined (group × [Ca\textsuperscript{2+}]\textsubscript{o} interaction effect, P = 0.75). Increasing [Ca\textsuperscript{2+}]\textsubscript{o} tended to lower the \( t_{\text{1/2}} \) of [Ca\textsuperscript{2+}]\textsubscript{i} decline (Table 3), although the changes observed did not reach statistical significance ([Ca\textsuperscript{2+}]\textsubscript{o} effect, P = 0.0769).

Table 3. Effects of PLM overexpression on cardiac myocyte [Ca\textsuperscript{2+}]\textsubscript{i} transients

<table>
<thead>
<tr>
<th>[Ca\textsuperscript{2+}]\textsubscript{o}, mM</th>
<th>Resting [Ca\textsuperscript{2+}]\textsubscript{i}, nM</th>
<th>Systolic [Ca\textsuperscript{2+}]\textsubscript{i}, nM</th>
<th>Diastolic [Ca\textsuperscript{2+}]\textsubscript{i}, nM</th>
<th>% Increase in Fluorescence Intensity Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GFP</td>
<td>PLM</td>
<td>GFP</td>
<td>PLM</td>
</tr>
<tr>
<td>0.6</td>
<td>122 ± 18 (24)</td>
<td>109 ± 7 (29)</td>
<td>207 ± 20</td>
<td>233 ± 11*</td>
</tr>
<tr>
<td>1.8</td>
<td>98 ± 5 (46)</td>
<td>91 ± 5 (40)</td>
<td>283 ± 14</td>
<td>255 ± 12*</td>
</tr>
<tr>
<td>5.0</td>
<td>104 ± 4 (38)</td>
<td>104 ± 5 (39)</td>
<td>359 ± 17</td>
<td>310 ± 11*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Nos. in parentheses are nos. of myocytes, without regard to no. of cells contributed by each heart. [Ca\textsuperscript{2+}]\textsubscript{o}, cytosolic Ca\textsuperscript{2+} concentration, measured 72 h after adenovirus infection. *P < 0.05, GFP vs. PLM.
PHOSPHOLEMMAN AND CARDIAC CONTRACTILITY

Overexpression of PLM in normal rat myocytes and postinfarction rat myocytes has been shown to be abnormal (3, 28, 30), we hypothesized that PLM might affect myocyte contractility by perturbing cardiac Ca\(^{2+}\) metabolism. To test this model, we have directed the overexpression of PLM in normal rat myocytes and measured its effects on contractility. We first established that under our continual pacing culture conditions, adult rat myocytes retained normal contractile function after 72 h of culture (Fig. 2), enough time for the exogenous PLM gene to be expressed and PLM protein to accumulate (Fig. 3). We also showed that the method of gene transfer by adenovirus infection had no effect on myocyte contractility (Fig. 2).

The first major finding of the present study is that PLM overexpression affected cardiac myocyte contractility (Fig. 4, Table 2). Specifically, under conditions that favored Ca\(^{2+}\) influx (0.6 mM [Ca\(^{2+}\)]\(_o\)), PLM myocytes contracted significantly more then GFP myocytes. Conversely, under conditions that favored Ca\(^{2+}\) efflux (5.0 mM [Ca\(^{2+}\)]\(_o\)), PLM myocytes shortened less than GFP myocytes. Assuming no changes in myofilament Ca\(^{2+}\) sensitivity with PLM overexpression, this complex pattern of contractile responses is consistent with the hypothesis that alterations in both Ca\(^{2+}\) influx and efflux pathways contribute to the different contractile behavior in PLM myocytes. Interestingly, the pattern of contractile abnormalities in PLM myocytes resembles that observed in postinfarction rat myocytes in that compared with sham-operated controls, contraction amplitudes in MI myocytes were higher at 0.6 mM but lower at 5.0 mM [Ca\(^{2+}\)]\(_o\) (28, 30). Thus our observations are consistent with the hypothesis that overexpression of PLM after infarction (21) may be causally related to development of contractile dysfunction.

If the differences in contractile behavior between GFP and PLM myocytes were in fact due to altered Ca\(^{2+}\) fluxes with PLM overexpression, then changes in [Ca\(^{2+}\)]\(_i\) transients during a stimulated twitch should be observed. This is indeed the case (Fig. 5; Table 3), and it constitutes the second major finding of the study. Similar to twitch amplitudes, compared with GFP myocytes, [Ca\(^{2+}\)]\(_i\) transient amplitudes were higher at 0.6 mM [Ca\(^{2+}\)]\(_o\) but lower at 5.0 mM [Ca\(^{2+}\)]\(_o\). The observation that [Ca\(^{2+}\)]\(_i\) transient amplitude differences between GFP and PLM myocytes mirror the pattern of contractile behavior differences supports our hypothesis that the abnormal contraction was caused by perturbed Ca\(^{2+}\) fluxes associated with PLM overexpression.

Overexpression of PLM was not associated with changes in protein levels of major cardiac Ca\(^{2+}\) transporters. Specifically, the levels of Na\(^{+}/Ca^{2+}\) exchanger, SERCA2, and calsequestrin were similar in GFP and PLM myocytes. In addition, SR Ca\(^{2+}\) uptake activity, as estimated by \(t_{1/2}\) of [Ca\(^{2+}\)]\(_i\) decline (26, 31), was not affected by PLM overexpression.

The mechanisms by which PLM perturbed Ca\(^{2+}\) fluxes and contractile function have not been addressed in the present exploratory study. However, the known properties of PLM measured in noncardiac cells provide a reasonable basis for speculation. PLM forms channels that are permeable to the zwitterion taurine (16, 17). Thus overexpression of PLM may lead to loss of taurine from cardiac myocytes. Taurine depletion is associated with myocardial contractile dysfunction (19), probably due to disordered contractile filaments and loss of myofibrillar bundles (13). However, the deleterious effects of taurine depletion on cardiac contractile elements should lead to reduction in contraction amplitudes at all [Ca\(^{2+}\)]\(_o\) levels (7). This is clearly not the case, because in PLM myocytes contraction amplitudes measured at 0.6 mM [Ca\(^{2+}\)]\(_o\), were higher than in GFP myocytes (Fig. 4; Table 3). An alternative mechanism by which taurine may affect myocyte contractility is perturbation of [Ca\(^{2+}\)]\(_i\) homeostasis by changing Ca\(^{2+}\) fluxes through the Na\(^{+}/Ca^{2+}\) exchanger (1). However, the effects of taurine on Na\(^{+}/Ca^{2+}\) exchanger function remain controversial (1, 7, 11).

Another potential mechanism by which PLM can affect contractility is by inhibiting Na\(^{+}/Ca^{2+}\) exchange.

**Fig. 5. PLM overexpression alters cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) transients in adult rat myocytes.** Isolated myocytes were loaded with the Ca\(^{2+}\) indicator fura 2, myocytes were paced (1 Hz) to contract at 37°C and [Ca\(^{2+}\)]\(_o\) of 0.6, 1.8, and 5.0 mM. Results are summarized in Table 3.
activity. The pattern of contractile and [Ca\(^{2+}\)] transient abnormalities observed in PLM myocytes (amplitudes higher at 0.6 mM [Ca\(^{2+}\)], but lower at 5.0 mM [Ca\(^{2+}\)]) mimics that observed in postinfarction rat myocytes (3, 28–31), in which Na\(^+/Ca\(^{2+}\)) exchange activity has been shown to be depressed (6, 33). In addition, the pattern of contraction and [Ca\(^{2+}\)] transient alterations in PLM myocytes is opposite to that observed in myocytes in which Na\(^+/Ca\(^{2+}\)) exchange activity was enhanced by overexpression (32). Specifically, in adult rat myocytes overexpressing the Na\(^+/Ca\(^{2+}\)) exchanger, both contraction and [Ca\(^{2+}\)] transient amplitudes were lower at 0.6 mM [Ca\(^{2+}\)], but higher at 5.0 mM [Ca\(^{2+}\)], compared with their controls (32). These two observations suggest that PLM probably regulates Na\(^+/Ca\(^{2+}\)) exchange activity, either directly or indirectly.

PLM belongs to the FXYD gene family of small ion transport regulators (23). This family includes the γ-subunit of Na\(^+\)-K\(^+\)-ATPase. The γ-subunit of Na\(^+\)-K\(^+\)-ATPase has been shown to bind to and modulate Na\(^+\)-K\(^+\)-ATPase activity (24). A potential mechanism by which PLM alters myocyte contraction is inhibition of Na\(^+\)-K\(^+\)-ATPase activity, thereby indirectly modulating Na\(^+/Ca\(^{2+}\)) exchange activity. Further support for this hypothesis is the observation that shark rectal glands contained a PLM-like protein (PLMS) that associated with the α-subunit of Na\(^+\)-K\(^+\)-ATPase (15). PLMS has homology to PLM and similar mobility on SDS-PAGE of 15 kDa (15). Unlike the γ-subunit of Na\(^+\)-K\(^+\)-ATPase, which lacked PLM’s basic COOH-terminal sequence with phosphorylation motifs (18), PLMS could be phosphorylated by both protein kinase A and protein kinase C (15). Phosphorylated PLMS dissociated from the α-subunit of Na\(^+\)-K\(^+\)-ATPase, with resultant activation of the enzyme (15). The close homology between PLM and PLMS suggests that PLM has homology between PLM and PLMS suggests that PLM could modulate Na\(^+\)-K\(^+\)-ATPase activity by association with the enzyme in cardiac myocytes, similar to the PLMS association with shark Na\(^+\)-K\(^+\)-ATPase. Thus it is possible that PLM could modulate Na\(^+\)-K\(^+\)-ATPase activity and thus indirectly affect Na\(^+/Ca\(^{2+}\)) exchange. Inhibition of Na\(^+\)-K\(^+\)-ATPase by PLM would be expected to increase intracellular Na\(^+\) and, based on thermodynamics considerations alone, should diminish forward Na\(^+/Ca\(^{2+}\)) exchange (Ca\(^{2+}\) efflux) but enhance reverse Na\(^+/Ca\(^{2+}\)) exchange (Ca\(^{2+}\) influx). This would result in increased contraction amplitudes in PLM myocytes studied under both low (Ca\(^{2+}\) efflux-promoting)- and high (Ca\(^{2+}\) influx promoting)-[Ca\(^{2+}\)]\(_i\) conditions, a prediction not consistent with our observations on myocytes overexpressing PLM. Therefore, mechanisms other than or in addition to sole inhibition of Na\(^+\)-K\(^+\)-ATPase by PLM needed to be invoked to fully explain our experimental observations. In this light, we should not overlook the possibility that PLM directly interacts with Na\(^+/Ca\(^{2+}\)) exchanger and modulates its activity. Much further work is required, however, to elucidate the mechanisms by which PLM affects cardiac contractility.

In summary, we have established an in vitro myocyte culture model system in which normal contractile function was preserved for at least 72 h. Infection with adenovirus had no effect on myocyte contractility. Overexpression of PLM by adenovirus-mediated gene delivery resulted in altered contraction and [Ca\(^{2+}\)] transient. Specifically, PLM myocytes contracted more than GFP myocytes at 0.6 mM [Ca\(^{2+}\)], but shortened less than GFP myocytes at 5.0 mM [Ca\(^{2+}\)]. This pattern of contractile abnormality is similar to that observed in postinfarction myocytes, in which contraction amplitudes were higher at 0.6 mM [Ca\(^{2+}\)], but lower at 5.0 mM [Ca\(^{2+}\)], compared with sham-operated myocytes (30). Na\(^+/Ca\(^{2+}\)) exchanger, calsequestrin, and SERCA2 expression and SR Ca\(^{2+}\) uptake activity were not affected by PLM overexpression. We conclude that overexpression of PLM resulted in perturbation of Ca\(^{2+}\) fluxes, with resultant contractile abnormalities in adult rat myocytes. We speculate that PLM overexpression may partly account for the contractile dysfunction in postinfarction myocytes.

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