Phospholemman-Phosphorylation Mediates the β-Adrenergic Effects on Na/K Pump Function in Cardiac Myocytes

Sanda Despa, Julie Bossuyt, Fei Han, Kenneth S. Ginsburg, Li-Guo Jia, Howard Kutchai, Amy L. Tucker, Donald M. Bers

Abstract—Cardiac sympathetic stimulation activates β-adrenergic (β-AR) receptors and protein kinase A (PKA) phosphorylation of proteins involved in myocardial Ca regulation. The Na/K-ATPase (NKA) is essential in regulating intracellular [Na] ([Na]i), which in turn affects [Ca]i via Na/Ca exchange. However, how PKA modifies NKA function is unknown. Phospholemman (PLM), a member of the FXYD family of proteins that interact with NKA in various tissues, is a major PKA substrate in heart. Here we tested the hypothesis that PLM phosphorylation is responsible for the PKA effects on cardiac NKA function using wild-type (WT) and PLM knockout (PLM-KO) mice. We measured NKA-mediated [Na]i decline and current (Ipump) to assess β-AR effects on NKA function in isolated myocytes. In WT myocytes, 1 μmol/L isoproterenol (ISO) increased PLM phosphorylation and stimulated NKA activity mainly by increasing its affinity for internal Na (Km decreased from 18.8 ± 1.4 to 13.6 ± 1.5 μmol/L), with no significant effect on the maximum pump rate. This led to a significant decrease in resting [Na]i (from 12.5 ± 1.8 to 10.5 ± 1.4 μmol/L). In PLM-KO mice under control conditions, Km (14.2 ± 1.5 μmol/L) was lower than in WT, but comparable to that for WT in the presence of ISO. Furthermore, ISO had no significant effect on NKA function in PLM-KO mice. ATPase activity in sarcoplasmic vesicles also showed a lower Km(Na) in PLM-KO versus WT (12.9 ± 0.9 versus 16.2 ± 1.5). Thus, PLM inhibits NKA activity by decreasing its [Na]i affinity, and this inhibitory effect is relieved by PKA activation. We conclude that PLM modulates the NKA function in a manner similar to the way phospholamban affects the related SR Ca-ATPase (inhibition of transport substrate affinity, that is relieved by phosphorylation). (Circ Res. 2005;97:252-259.)

Key Words: Na pump ▪ phospholemman ▪ signal transduction ▪ ion channels

Activation of the sympathetic nervous system and cardiac β-adrenergic (β-AR) receptors causes cAMP formation and activation of protein kinase A (PKA). In cardiac myocytes, PKA phosphorylates several targets with key roles in the control of excitation–contraction coupling (ECC), including L-type Ca2+ channels, phospholamban (PLB) and troponin-I, as well as other sarcolemmal proteins such as voltage-gated Na and K channels and phospholemman (PLM).

During sympathetic activation, the larger Ca influx via more frequent and larger Ca current must be balanced by enhanced Na extrusion via the Na/Ca exchange (NCX) that is driven by larger Ca transients. This increases Na influx at each beat, along with more frequent and larger Na current, which increases intracellular [Na] ([Na]i). To limit the rise in [Na]i, the greater Na influx must be compensated for by an enhanced Na extrusion via the Na/K pump (NKA). Indeed, early studies indicated stimulation of the Na-pump by β-AR activation.1-3 However, there is controversy at present because some recent studies in single myocytes using NKA pump current (Ipump) found either stimulation,4-6 inhibition,7,8 or no change8 in Ipump on β-AR stimulation.

Controversy extends beyond the direction of β-AR effects on NKA function, as to the molecular mechanism involved. NKA α subunit can be phosphorylated by PKA only in the presence of detergents or after reconstitution,10,11 whereas in situ the phosphorylation site may be inaccessible to the kinase.12 This raises the question of whether phosphorylation of a NKA regulatory protein could mediate β-AR effects.

In heart, such a role could be played by the small transmembrane protein PLM,13,14 long known to be a major cardiac substrate for both PKA and protein kinase C (PKC).15,16 However, the physiological role of PLM is poorly understood. Recent studies show that PLM associates with NKA and decreases its apparent Na and K affinity.13 Furthermore, PLM is one of the 7 members of the FXYD gene family, which also includes the NKA γ-subunit and CHIF in the kidney, both of which associate with and modulate NKA.17 However, PLM is the only FXYD protein that is known to be phosphorylated. Thus, PLM might affect NKA function in a manner similar to the way PLB affects SERCA, a P-type pump closely related to NKA, ie, inhibition, relieved by phosphorylation.
The aim of this article is to test the hypotheses that (1) β-AR stimulation activates NKA by increasing its [Na]i affinity, (2) PLM inhibits NKA activity in a manner analogous to PLB inhibition of SERCA (by decreasing [Na]i affinity), and (3) β-AR stimulation of NKA is mediated by PLM phosphorylation. We combined measurements of NKA-mediated [Na]i decline and Ipump to determine the effect of β-AR activation by isoproterenol (ISO) on NKA function in single myocytes isolated from wild-type (WT) mice and mice in which the PLM gene was targeted (PLM-KO).18 ISO stimulated the pump in the WT mice by reducing the Km for NaCl, 2 EGTA, 1 MgCl2, 10 HEPES, and 10 glucose (pH 7.4). NCX dependence was checked to assure that these conditions measure Na/K pump rather than inward current attributed to CFTR. We showed previously that the K-sensitive current is equivalent to ouabain-sensitive current.20

**Materials and Methods**

A more detailed Materials and Methods is available in the online data supplement at http://circres.ahajournal.org.

**Generation of PLM-KO Mice**

PLM-KO mice were generated at the University of Virginia (Charlottesville, Va) as previously described19 except that they are now congenic on a pure C57B/6 background. Heterozygous breeding pairs were used to generate PLM-KO and WT littermates. Mice of 3 to 4 months of age were used, before PLM-KO mice developed hypertrophy.18 All animal protocols were approved by the Animal Care and Use Committee at Loyola University Chicago and University of Virginia.

**Myocyte Isolation**

Isolation of mouse ventricular myocytes was as previously described.19 Briefly, PLM-KO mice and age-matched WT littermates were anesthetized (with 3% to 5% isoflurane). Hearts were excised quickly and mounted on a Langendorff perfusion apparatus and exposed to 0.8 mg/mL collagenase (type B, Boehringer Mannheim) for 7 to 12 minutes. Ventricular tissue was removed, dispersed, filtered, and myocyte suspensions were rinsed several times. The yield of viable rod-shaped myocytes was 70% to 80%. Cell size was similar in WT and KO myocytes based on membrane capacitance (204 ± 14 versus 215 ± 20 pF, respectively, n=8 each).

**Intracellular [Na] Measurements in Intact Myocytes**

Isolated myocytes plated on laminin-coated coverslips were loaded with SBFI-AM, and dual excitation fluorescence measurements (at 340 and 380 nm; F340/F380) were performed after background subtraction and converted to [Na]i using calibration at the end of each experiment in the presence of 10 μmol/L gramicidin and 100 μmol/L strophanthidin.21 Generally, [Na]i was measured at 15 to 60 s intervals to minimize indicator photobleaching and cell photodamage. All the measurements were at room temperature.

**Na Efflux Through the Na/K-Pump**

Na/K pump flux was determined as the rate of pump-mediated [Na]i decline.21 Myocytes were Na-loaded by inhibiting the Na/K pump in a K-free solution containing (mmol/L): 145 NaCl, 2 EGTA, 10 HEPES, and 10 glucose (pH=7.4). [Na]i decline was measured on pump reactivation in solution containing (mmol/L): 140 TEA-Cl, 4 KCl, 2 EGTA, 1 MgCl2, 10 HEPES, and 10 glucose (pH=7.4). NCX is blocked in this Na-free, Ca-free solution, assuming that measured PLM effects on [Na]i decline depend directly on NKA and not secondary to any effect on NCX. Because cell volume does not change with this protocol,21 [Na]i decline reflects Na efflux. The rate of [Na]i decline (−d[Na]i/dt) was plotted versus [Na], and fitted with:

\[
-\frac{d[Na]}{dt} = \frac{V_{max}}{1 + ([Na]/Km)^n}
\]

**Simultaneous Na/K Pump Current and [Na]i Measurements**

Simultaneous Ipump and [Na]i measurements were done as previously described.20 Briefly, thapsigargin pretreated myocytes were whole-cell voltage-clamped under conditions that minimized the effect of cellular dialysis by the patch pipette on [Na], while maximizing its compatibility with sarcolemmal Na fluxes. Electrogate series resistance (3 to 4 MΩ before patching) was 6 to 12 MΩ during current recording. The standard pipette solution contained (mmol/L): 10 NaCl, 20 KCl, 100 K-aspartate, 20 TEA-Cl, 10 HEPES, 5 Mg-ATP, 0.7 MgCl2 (≈1 mmol/L free Mg), 3 BaPTA, 1.5 CaCl2 (≈100 mM free Ca), 1 SBFI tetramassium salt, pH=7.2. The external solution contained (mmol/L): 136 NaCl, 5 NiCl2, 2 BaCl2, 1 MgCl2, 5 HEPES 10 glucose, and 4 KCl (4 mmol/L K solution) or Tris-Cl (0 mmol/L K solution), pH=7.4. Ipump was measured at −20 mV as the outward shift induced by switching from K-free to K-containing external solution. This also corrected for a small variable ISO-stimulated current attributed to CFTR. We showed previously that the K-sensitive current is equivalent to ouabain-sensitive current.20

**3H-Ouabain Binding**

3H-ouabain binding was measured with a filtration method using isolated myocytes.23,24 Briefly, isolated myocytes were permeabilized (≈25 μg/mL saponin) and incubated at 37°C for 6 hours in solution containing (mmol/L) 0.1 [3H]ouabain, 0.01 sodium meta-vanadate, 5 MgCl2, 50 Tris-HCl (pH 7.4), 5 Tris-PO4. After incubation myocytes were filtered (Whatman GF/C filter paper) and washed, and filter-associated radioactivity was determined by liquid scintillation counting. Non-specific [3H]ouabain was determined by using an excess of unlabelled ouabain (1 mmol/L) and [ouabain] dependence was checked to assure that these conditions measure >90% of the number of Na/K-ATPase sites (Bmax).

**Sarcolemmal Isolation and ATPase Activity**

A sarcolemmal-enriched fraction was prepared as described25 with modifications (see online data supplement). Ouabain-sensitive Na/K-ATPase activity was measured at 37°C in a spectrophotometric enzyme-coupled assay as described.26 Sarcolemmal (≈2 μg) was incubated in medium containing (mmol/L) Tris-HCl 30, KCl 20, MgCl2 3, EDTA 1, Phospho(enol)pyruvate 15, NADH 1, ATP 3, ±ouabain 10, and 4.6 U of lactic dehydrogenase and 3.3 U of pyruvate kinase at pH 7.2, with [NaCl]=0 to 120 mmol/L. The decline in NADH absorbance at 340 nm is used to calculate ATPase rate sensitive to 10 mmol/L ouabain.

**Statistical Analysis**

Data are expressed as mean±SEM. Student t test (paired when appropriate) was used for statistical discriminations, with P<0.05 considered significant.

**Results**

Effect of ISO on the Na/K Pump-Mediated Na Efflux in Myocytes From WT and PLM-KO Mice

The effect of β-AR stimulation on NKA function was measured in intact myocytes by measuring the [Na]-
dependence of NKA-mediated Na efflux. Myocytes were Na-loaded by incubation in K-free solution to block NKA (Figure 1A). Then the Na/K-Pump was reactivated by readmission of 4 mmol/L [K]o (and removal of extracellular Na) and the time course of [Na]i decline was measured. The protocol was then repeated in the presence of 1 μmol/L ISO (Figure 1A). In the experiment shown in Figure 1A, Na efflux in Na-free solution is mediated by both NKA and other mechanisms (outward leak). To account for the leak, we performed a parallel series of experiments where Na efflux was measured in the presence of 10 mmol/L ouabain, to completely block NKA. [Na]i decline in each case was numerically differentiated and d[Na]i/dt was plotted as a function of [Na]i (Figure 1B). NKA-mediated Na efflux was obtained by subtracting the leak component from the total Na efflux. When 2 consecutive control runs are done, the resulting Na efflux is highly reproducible (see online Figure IS).

Figure 2 shows average data for the effect of ISO on the [Na]i-dependence of Na extrusion by the Na/K pump in WT (n=10 cells, 4 hearts) and PLM-KO mice (n=9 cells, 4 hearts) with and without 1 μmol/L ISO. Data were normalized with respect to Vmax (mean fit parameters are in the Table).

Vmax, Km, and nHill under control conditions were similar to the WT in the presence of ISO (Table). Thus β-AR stimulation makes the [Na]i-dependence of NKA in myocytes from WT mice similar to that found in the PLM-KO mice. Furthermore, ISO failed to significantly shift Km in the PLM-KO mice (14.6±1.9 versus 13.1±1.5 mmol/L, n=9, P=0.611). ISO did not significantly alter the Vmax or nHill in either WT or PLM-KO myocytes.

The [Na]i-dependence of ouabain-sensitive ATPase in sarcolemmal vesicles was also shifted in PLM-KO versus WT ventricle (Km=12.9 versus 16.2 mmol/L; Table). This is similar to the shift based on d[Na]i/dt in myocytes (Km=14.2 versus 18.8 mmol/L; Table) and confirms the PLM-induced inhibition of apparent Na affinity in a more controlled (but less physiological) environment.

Effect of ISO on I_pump in Myocytes From WT and PLM-KO Mice

To further test our hypotheses, we determined the effect of β-AR stimulation on I_pump by simultaneously monitoring I_pump and [Na]i under voltage clamp conditions at [Na]i in the range of the Km, where differences should be most apparent. In ruptured patch whole-cell configuration, NKA was inhibited for 10 to 12 minutes in a K-free solution (Figure 3). This led to a rise in [Na]i above that in the pipette solution caused by Na entry from the external solution. Then, the pump was abruptly reactivated by restoring K (4 mmol/L) while measuring membrane current (Figure 3A and 3D) and [Na]i (Figure 3B and 3E). I_pump decays (Figure 3A and 3D) as [Na]i,
[Na]-dependence of NKA-mediated Na extrusion in myocytes from WT (10 Cells, 4 Hearts) and PLM-KO Mice (9 Cells, 4 Hearts), and NKA-mediated ATPase activity in WT vs PLM-KO plasmalemma-enriched fractions (4–9 hearts were pooled and 9–10 separate series of ATPase assays were run)

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>WT + ISO</th>
<th>PLM-KO</th>
<th>PLM-KO + ISO</th>
</tr>
</thead>
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<tr>
<td>[d(Na]/dt in isolated ventricular myocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V_{max} (mM/min)</td>
<td>5.6±0.7</td>
<td>6.3±0.6</td>
<td>6.4±0.7</td>
<td>7.1±0.8</td>
</tr>
<tr>
<td>K_H (mM [Na])</td>
<td>18.8±1.4</td>
<td>13.6±1.5</td>
<td>14.2±1.5†</td>
<td>13.1±1.4</td>
</tr>
<tr>
<td>n_Hill</td>
<td>2.8±0.1</td>
<td>2.9±0.1</td>
<td>2.9±0.1</td>
<td>2.7±0.1</td>
</tr>
</tbody>
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Na/K-ATPase activity in sarcolemmal fraction

|                          |         |          |         |              |
| V_{max} (μmol·mg⁻¹·min⁻¹) | 1.47±0.20| 1.22±0.0016 |        |              |
| K_H (mM [Na])             | 16.2±1.5 | 12.9±0.9† |        |              |
| n_Hill                    | 1.5±0.1 | 1.4±0.1  |        |              |

*Significantly different ISO vs control; †significantly different PLM-KO vs WT

declines (Figure 3B and 3E). The protocol was repeated in the same cell in the presence of 1 μmol/L ISO. Figure 3A through 3C shows a representative experiment in myocytes from WT mouse. I_{pump} is larger in the presence of ISO, which results in a faster decrease in [Na]. The plot of I_{pump} versus [Na], (Figure 3C) emphasizes that I_{pump} is higher in the presence of ISO for a given [Na], in a range (9 to 14 mmol/L) comparable to the K_H. In contrast, ISO has no effect on I_{pump} in myocytes from PLM-KO mouse (Figure 3D through 3F). Thus, I_{pump} data are consistent with a PLM-dependent stimulation of NKA function by ISO, via an increased affinity for internal Na.

Figure 3 shows pooled data from experiments like that in Figure 3. For an initial [Na] that is not significantly different on I_{pump} reactivation in any of the 4 cases (Figure 4A; ~14.5 mmol/L) mean peak I_{pump} is significantly increased by ISO in WT but not PLM-KO myocytes. This further confirms that ISO stimulation of NKA depends on the presence of PLM.

Figure 4 shows pooled data from experiments like that in Figure 3. For an initial [Na] that is not significantly different on I_{pump} reactivation in any of the 4 cases (Figure 4A; ~14.5 mmol/L) mean peak I_{pump} is significantly increased by ISO in WT but not PLM-KO myocytes. This further confirms that ISO stimulation of NKA depends on the presence of PLM.
Phosphorylation of Phospholemman on β-AR Stimulation and PLM-NKA Association

Figure 5A shows that PLM was indeed undetectable in PLM-KO myocytes, but there was also downregulation of NKA expression (assessed by an antibody that detects all 3 NKA isoforms equally). This might explain why IPump in ISO-stimulated WT myocytes is larger than in PLM-KO cells (Figure 4B). Immunoblots with phospho-specific antibodies (Figure 5B) show an increase in PLM phosphorylation at Ser-68 (PKA site), but not at Ser-63 (the PKC site) in myocytes exposed to ISO for 15 minutes. Pooled data from Western blots indicated a 20% reduction in NKA expression (Figure 5C), and this was very similar to the 25% reduction in B_max detected in 3H-ouabain binding assays (Figure 5D).

Thus, in mice without tonic PLM-dependent NKA inhibition (which would increase NKA activity), there appears to be an adaptation to reduce the number of Na-pumps. This smaller number of Na-pumps in the PLM-KO and their inability to be regulated by β-AR activation would limit the ability of cells to increase Na extrusion in response to sympathetic activation.

Figure 5E shows that PLM and NKA α1-subunit coimmunoprecipitate. Thus, there is both a physical association and functional interaction between PLM and NKA in mouse ventricular myocytes.

Effect of ISO on Resting [Na]i in Myocytes From WT and PLM-KO Mice

Figure 6 shows resting [Na]i in myocytes from WT and PLM-KO mice. A, Representative traces for WT and PLM-KO mice; B, Mean data from 8 WT (4 hearts) and 10 PLM-KO myocytes (4 hearts). *P<0.05.

Figure 6. Effect of 1 μmol/L ISO on the resting [Na]i in myocytes from WT and PLM-KO mice; (B) Mean data from 8 WT (4 hearts) and 10 PLM-KO myocytes (4 hearts). *P<0.05.
Discussion

The present study demonstrates that PLM regulates the Na/K pump in a manner similar to the way PLB modulates SERCA, ie, it inhibits the pump by decreasing the affinity for intracellular Na ($K_m=14.2\pm1.5$ mmol/L in PLM-KO mouse versus $18.8\pm1.4$ mmol/L in WT) and the inhibition is relieved on PLM phosphorylation ($K_m=13.6\pm1.5$ mmol/L in WT mice with ISO). Furthermore, the lack of NKA stimulation by ISO in PLM-KO mouse indicates that β-AR effects on NKA are mediated primarily by PLM, rather than direct phosphorylation of NKA.

β-AR Stimulation and NKA Activity

Historically, numerous studies indicated that β-AR stimulation enhances NKA activity and lowers [Na]i in heart (eg, see references in Glitsch et al12). However, there is recent controversy. Most studies at the single cell level, using either $I_{\text{Pump}}$ (voltage-clamp) or [Na]i measurements indicate that NKA function is increased by β-AR stimulation (present study3,4,25). However, some voltage-clamp studies have reported either no effect of β-AR on $I_{\text{Pump}}$ in rat or even an inhibition of guinea-pig $I_{\text{Pump}}$ that was [Ca]i-dependent (decreased $I_{\text{Pump}}$ when pipette [Ca] was <150 nM, but increased $I_{\text{Pump}}$ at high pipette [Ca]).28 It has also been reported that β-AR stimulation of $I_{\text{Pump}}$ only affects the αi isofrom of NKA (the major form in most hearts), but not αi,6,28.

Both $I_{\text{Pump}}$ and [Na]i measurements have advantages and disadvantages in assessing the effect of β-AR stimulation on NKA. [Na]i measurements, especially using fluorescent indicators, are less invasive as they are done in intact cells without altering the cellular environment. However, the rate of [Na]i change (d[Na]i/dt) is an indirect measure of Na-pump flux that could be affected by changes in cellular Na buffering or cell volume (although neither is expected to change during β-AR stimulation). In $I_{\text{Pump}}$ measurements, cell dialysis by the patch pipette can partly control the intracellular environment, but also alter intracellular composition (eg, of important messengers) and can be subject to contaminating ionic currents.

Here, we found evidence for stimulation of NKA function by β-AR in 3 contexts: (1) in nondialyzed myocytes (as the [Na]i-dependence of pump-mediated [Na], decline on abrupt NKA re-activation), (2) in cells under voltage clamp (simultaneous $I_{\text{Pump}}$ and [Na]i decline measurements), and (3) in intact quiescent myocytes at physiological levels of [Na]i and [K]. Our data in intact WT mouse ventricular myocytes indicate that β-AR activation stimulates Na-pump activity by increasing the NKA affinity for internal Na without significantly altering maximal NKA rate (Figure 2A and Table). This conclusion is supported by the $I_{\text{Pump}}$ and [Na]i decline results in voltage-clamped cells, as they show an increased $I_{\text{Pump}}$ for a given [Na], near the $K_m$ in the presence of ISO (Figures 3 and 4). β-AR–induced NKA stimulation is also consistent with the decline in resting [Na]i on ISO stimulation in otherwise unperturbed myocytes (Figure 6).

PLM Modulation of NKA Function

PLM, a 72–amino acid sarcolemmal protein expressed highly in heart and brain, is a member of the FXYD protein family (named for a conserved Pro-Phe-X-Tyr-Asp motif).29 FXYD proteins have a single membrane span and include the NKA γ-subunit (FXYD-2), the regulator of renal NKA (FXYD-4, or CHIF), and the PLM-like shark rectal gland protein (PLMS). PLM, FXYD-2, -4, -7, and PLMS all coimmunoprecipitate with NKA α subunits and modulate NKA function,6,13,30–33 but how PLM regulates cardiac NKA is unclear. Cramb et al13 showed that, when transfected into Xenopus oocytes, PLM associates specifically (stoichiometrically) and stably with rat NKA α1 and α2 subunits and that PLM inhibits NKA by reducing its apparent affinity for [Na]i ($K_m$ increased from 9.3 to 16.5 mmol/L) and [K]o ($K_m$ increased from 0.49 to 0.67 mmol/L).

Initial data in PLM-KO mice showed greater reduction of maximal Na/K-ATPase activity in a sarcolemmal-enriched fraction than for NKA expression in homogenates (~20%),18 implying that PLM expression might activate NKA. Here we found a similar degree of downregulation of NKA α subunit in PLM-KO based on myocyte β-H-ubain binding (25%), myocyte Western blot (20%), and membrane fraction Na/K-ATPase (17%, nonsignificant), but no decrease in $V_{\text{max}}$ for d[Na]i/dt in PLM-KO versus WT myocytes (Table). Possibly the stronger depression of plasmaemmal Na/K-ATPase $V_{\text{max}}$ reported in the prior study18 was complicated by membranes from nonmyocytes, differential sarcolemmal enrichment, or some other assay factor.

Our results here with PLM-KO mice indicate that PLM depresses NKA activity, mainly via reduced [Na]i affinity (by ~4 mmol/L), but an effect of PLM on $V_{\text{max}}$ cannot be ruled out. That is, PLM-KO myocytes had reduced NKA protein expression (20% to 25%) with a tendency toward higher $V_{\text{max}}$ without apparent changes in $K_m$ (12 mmol/L, but increased [Na]i at high pipette [Ca]).28 It has also been reported that β-AR stimulation of $I_{\text{Pump}}$ only affects the αi isofrom of NKA (the major form in most hearts), but not αi,6,28.

Despite NKA inhibition by PLM, resting [Na]i is not higher in myocytes from WT versus PLM-KO mice. This might be attributable in part to the reduced NKA expression in PLM-KO mice (Figure 5). The similar resting [Na]i in WT and PLM-KO myocytes could also be partly attributable to increased Na influx in PLM-KO mice. Using the initial rate of [Na]i, rise on NKA blockade (as in Figure 1) the mean apparent Na influx rate was slightly higher in PLM-KO versus WT myocytes (3.1±0.5 versus 2.7±0.4 mmol/L/min at 12 mmol/L [Na]i, n=14), but the difference was not significant.
PLM Phosphorylation
PLM is unique in the FXYD family in having multiple cytosolic phosphorylation sites and is a major cardiac target for β-AR-mediated phosphorylation. Quantitatively, PLM phosphorylation by PKA is comparable to that of troponin I and PLB. PLM is phosphorylated by ISO under the conditions used here (Figure 5). In PLM-KO mice ISO did not significantly alter either V_{\text{max}} and K_m of the Na/K pump or resting [Na]. This is most consistent with the β-AR–dependent NKA stimulation in WT mice being mediated by PLM phosphorylation. After β-AR stimulation NKA function in WT mice is almost identical to that in PLM-KO mice without ISO. Thus, PLM inhibits NKA (by decreasing its affinity for [Na]), and this inhibition is relieved on PLM phosphorylation. This is highly analogous to the way PLB modulates SERCA activity.

Interestingly, the association between PLM and NKA, as determined by coIP experiments, appears to be unaffected by PKA phosphorylation. Thus phosphorylation may change the PLM-NKA interaction but does not necessarily result in a complete dissociation. In the analogous PLB-SERCA system it was long thought that PLB phosphorylation caused it to dissociate from SERCA, but more recent results show that PLB remains bound to SERCA even after phosphorylation and abolition of SERCA inhibition.

Physiological Context of β-AR Activation of NKA
During β-AR stimulation in the sympathetic fight or flight response, Na influx into cardiac myocytes is greatly increased. This is attributable to more frequent Na current (which may also be larger caused by PKA) and more Na entry via NCX, that is both because of higher frequency and because larger Ca transients drive greater Ca extrusion coupled Na influx at each beat. Looked at another way, the higher Ca current on β-AR stimulation increases Ca influx, and Ca extrusion (coupled to Na influx via NCX) must also be increased to attain Ca flux balance. Indeed, Na entry via NCX is a major fraction of Na influx during the cardiac cycle. Enhancement of NKA activity may thus be an integral part of the sympathetic response of the heart to enhance Na extrusion to better keep up with the higher level of Na influx. This may limit the rise in [Na], that occurs during the combined inotropic and chronotropic effects on the heart. Our data show that NKA stimulation by β-AR activation results in reduced [Na] in resting myocytes from WT but not PLM-KO mice (Figure 6). The inability of NKA to be stimulated by β-AR in the PLM-KO mouse could lead to excessive elevation of [Na], during sympathetic activation, which might have both the benefits and the risks associated with NKA inhibition by glycosides (inotropy, but enhanced arrhythmogenesis). This process might be further complicated by possible direct effects of PLM on NCX.

In summary, we have shown that (1) β-AR stimulation activates NKA in mouse cardiac myocytes by increasing its affinity for internal Na, with no significant effect on the maximum pump rate, (2) PLM inhibits NKA activity by decreasing its [Na] affinity, and (3) β-AR stimulation of NKA is mediated by PLM phosphorylation. Thus, our data indicate that PLM modulates the NKA function in a manner similar to the way PLB affects SERCA.

Acknowledgments
This work was supported by grants from the National Institutes of Health (HL-30077, HL-64724; D.M.B.), the Cardiovascular Research Center at University of Virginia (A.L.T.), and fellowships from the American Heart Association (S.D., J.B.). The authors thank Brian French and Jaime O’Brien for myocyte preparation.

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Circ Res. 2005;97:252-259; originally published online July 7, 2005;
doi: 10.1161/01.RES.0000176532.97731.e5
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Materials and Methods

Generation of PLM-KO mice

PLM-KO mice were as previously described except that they are now congenic on a pure C57B/6 background. Briefly, a PLM-KO mouse cell line was created by replacing the PLM gene (exons 3-5) with an insert containing lacZ in the AB2.2 stem cell line. Blastocyst injection and generation of germ-like chimeric mice were performed in the University of Virginia Transgenic Facility. Heterozygous breeding pairs were used to generate PLM-KO and WT littermates. Mice of 3-4 months old were used, before PLM-KO mice developed hypertrophy at 5-6 months. All animal protocols were approved by the Animal Care and Use Committee at Loyola University Chicago and University of Virginia.

Myocyte isolation

Isolation of mice ventricular myocytes was as previously described. Briefly, PLM-KO mice and age-matched WT littermates were anesthetized in a gas chamber with 3-5% isoflurane (with the remainder O₂). Hearts were excised quickly and mounted on a gravity-driven Langendorff perfusion apparatus. Hearts were perfused for 6 min at 37°C with nominally Ca-free medium (DMEM) with 24 mmol/L NaHCO₃ bubbled with 95%-O₂:5%-CO₂ (pH 7.4). Perfusion was then switched to the same solution containing 0.8 mg/ml collagenase (type B, Boehringer Mannheim, Indianapolis, IN). When the heart became flaccid (7-12 min), ventricular tissue (both left and right) was removed, dispersed, filtered, and myocytes suspensions were rinsed several times. The yield of viable, rod-shaped myocytes was 70-80%. The cell size, as indicated by membrane capacitance, was similar in the WT and KO myocytes (204±14 pF vs. 215±20 pF, n=8 for both WT and KO).

Intracellular [Na] measurements in intact myocytes

Isolated myocytes were plated on laminin-coated coverslips and incubated with 10 µmol/L SBFI-AM and Pluronic F-127 (0.05% w/v) for 90 min at room temperature. After washing out the external dye, SBFI-AM was allowed to deesterify for 20 min. The normal Tyrode’s solution (NT) contained (mmol/L): 140 NaCl, 4 KCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES and 10 glucose (pH=7.4). Fluorescence was elicited by illumination with a 75 W xenon lamp. Dual excitation measurements (at 340 and 380 nm; F₃40 and F₃80) were performed as previously described. The emitted fluorescence was recorded at 535±20 nm. F₃40/F₃80 was calculated after background subtraction and converted to [Na], by calibration at the end of each experiment (using divalent-free solutions with 0, 10 or 20 mmol/L extracellular [Na]) in the presence of 10 µmol/L gramicidin and 100 µmol/L strophantidin. Generally, [Na], was monitored at 15-60 s intervals to minimize the photobleaching of the indicator and the photodamage of the cells. All the measurements were at room temperature.

Na efflux via Na/K-Pump

Na/K pump flux was determined as the rate of pump-mediated [Na], decline. Myocytes were Na- and K-loaded by inhibiting the Na/K pump in a K free solution containing (mmol/L): 145 NaCl, 2 EGTA, 10 HEPES and 10 glucose (pH=7.4). Because this solution is free of divalent cations, Na enters the cells via Ca channels and NCX leading to a [Na], rise by 30-35 mmol/L within 15-20 min. [Na], decline was measured upon pump reactivation in solution containing (mmol/L): 140 TEA-Cl, 4 KCl, 2 EGTA, 10 HEPES and 10 glucose (pH=7.4). NCX is blocked in this solution (0Na, 0 Ca conditions), which assures that any effect of PLM on [Na], decline is mediated by KNA and is not secondary to an effect on NCX. Since cell volume does not change with this protocol, [Na], decline reflects Na efflux. The rate of [Na], decline (−d[Na]/dt) was plotted vs. [Na], and fitted with: \( I_{\text{pump}} = V_{\text{max}} / (1 + (K_i/[Na])^n_{\text{Hill}}) \).

Simultaneous Na/K pump current and [Na], measurements

Simultaneous \( I_{\text{pump}} \) and [Na], measurements were done as previously described. Briefly, myocytes were whole-cell voltage clamped under conditions that minimized the effect of cell dialysis by the patch pipette on [Na], while maximizing its control by transsarcolemmal Na fluxes. The electrodes had a resistance of 3-4 MΩ when filled with the internal solution, resulting in a series resistance of 6-12 MΩ. Myocytes were pre-treated with thapsigargin (1 µmol/L, for 10 min) to empty the SR. The standard pipette solution contained (in mmol/L): 20 NaCl, 20 KCl, 100 K-aspartate, 20 TEA-Cl, 10 HEPES, 5 Mg-ATP, 0.7 MgCl₂ (=1 mmol/L free Mg), 3 BAPTA, 1.15 CaCl₂ (=100 nM free Ca), 1 SFI tetraammonium salt, pH=7.2. The external solution contained (in mmol/L): 136 NaCl, 5 NiCl₂, 2 BaCl₂, 1 MgCl₂, 5 HEPES 10 glucose, and 4 KCl (4 mmol/L K solution) or Tris-Cl (0 mmol/L K solution), pH=7.4. \( I_{\text{pump}} \) was measured at −20 mV (to inactivate Na channels) as the outward shift induced by switching from K free to K- and Na-containing external solution. We showed previously that this current is equivalent to ouabain-sensitive current. Some cells exhibited a detectable ISO-stimulated outward current that was insensitive to either ouabain or 4 mmol/L [K], (not shown), which we assume to be CFTR-mediated Cl current. This background current was comparable in WT and PLM-KO myocytes (0.03±0.01 vs. 0.05±0.04 A/F). This does not, however, rule out a possible effect of PLM on Cl fluxes and \( I_{\text{CFTR}} \). This is because \( I_{\text{CFTR}} \) was rather small, probably because the current was measured at −20 mV, close to the reversal potential for Cl.
the K-stimulated current as \( I_{\text{pump}} \) also corrects for this small variable ISO-activated current.

**Immunoblot**

Isolated myocytes were lysed in ice-cold buffer containing 1% NP-40 and (in mmol/L) 150 NaCl, 10 Tris (pH 7.4), 2 EGTA, 50 NaF, 0.2 NaVO₃ and protease inhibitors (Calbiochem). Lysates were then flash frozen and stored at -80°C. Lysates were also prepared after myocyte treatment with 1 \( \mu \)mol/L ISO. Standard Western blots were performed. In brief, lysates were size-fractionated on SDS-PAGE (10% for Na/K ATPase, 15% for PLM). Proteins were transferred to 0.20 \( \mu \)m nitrocellulose membranes and blocked in 5% nonfat dry milk in tris-buffered saline with Tween-20 (0.05%) for 2 hrs at room temperature. The blots were then incubated overnight at 4°C with primary antibody: pan-specific probe for Na/K ATPase a-subunits (a5, 1:100 dilution, Developmental Studies Hybridoma Bank), or custom-made PLM antibodies (1:5000 dilution) that detect PLM phosphorylated at Ser 68 (PKA and PKC site; CP68) or at Ser 63 (PKC site; CP63) and also one that prefers the non-phosphorylated form (PLM-C2).\(^9\) Equal protein loading was confirmed by reprobing with GAPDH.

**Immunoprecipitations**

NKA \( \alpha \) subunit and PLM were immunoprecipitated as previously described,\(^3\), using \( \alpha \)-isoform-specific antibodies (Upstate) and the PLM-C2 antibody, respectively. Cardiac membranes from isolated myocytes were solubilized for 30 min at 4°C using 6 mg/ml n-dodecyl octaethylene glycol monooctyl ether (C\(_{12}\)E\(_8\); Calbiochem, La Jolla, CA) in a buffer containing (in mmol/L): 140 NaCl, 25 imidazole, and 1 EDTA, pH 7.3. The extract was centrifuged for 30 min at 20,000\( \times \)g at 4°C to remove debris, after dilution with an equal volume of detergent-free buffer to 1mg/ml cardiac protein. The residual NKA in the pellet was found to be very low. The supernatant was incubated with primary antibodies or control IgG (1-2 \( \mu \)g/ml) overnight at 4°C with end-over-end rotation. Immunoprecipitates were collected after 2 hr of incubation with primary antibodies or control IgG (1-2 \( \mu \)g/ml) overnight at 4°C with end-over-end rotation. Immune complexes were collected by centrifugation at 10,000\( \times \)g for 10 min at 4°C and washed five times with solubilization buffer containing 0.05% C\(_{12}\)E\(_8\). After the final wash, the pellet was resuspended in sample buffer. Samples were centrifuged at 10,000\( \times \)g for 10 min and supernatants were saved to load on the gel.

\(^{3}\)H - Ouabain binding

\(^{3}\)H-ouabain binding was measured with a filtration method using isolated myocytes.\(^8,10\) Briefly, isolated myocytes permeabilized (~25 \( \mu \)g/ml saponin) were incubated at 37°C for 6 h in solution containing 100 \( \mu \)M \(^{3}\)H-ouabain, 10 \( \mu \)M sodium meta-vanadate, 5 mM MgCl₂, 50 mM Tris-HCl (pH 7.4), 5 mM Tris-PO₄. Following incubation myocytes were filtered (Whatman GF/C filter paper), washed five times with cold water and filter-associated radioactivity was determined by liquid scintillation counting. Non-specific \(^{3}\)H-ouabain was determined by using an excess of unlabeled ouabain (1 mM) and [ouabain] dependence was checked to assure that these conditions measure >90% of the number of Na/K-ATPase sites (\( B_{\text{max}} \)).

**Sarcolemmal isolation and ATPase activity**

Sarcolemmal-enriched fraction was prepared\(^1\) on ice or at 4°C. Mouse hearts (4-9) were pooled, minced and subjected to Polytron homogenization (for 3 bursts of 10 s each) in homogenizing buffer containing 0.25 M Sucrose, 20 mM Tris, 1 mM EDTA at pH 7.5, and then subjected to 20-30 strokes of a Dounce homogenizer. The homogenate was centrifuged at 14,000\( \times \)g for 10 min at 4°C. The supernatant was collected and reserved. The pellet was resuspended in the same buffer and subjected to 20-30 more strokes of a Dounce homogenizer and the 10 min, 14,000\( \times \)g centrifugation was repeated. The combined supernatants were then centrifuged at 44,000\( \times \)g to collect a microsomal pellet fraction for sucrose flotation. The pellet was resuspended in 0.5 ml 1 M sucrose, over which were layered 0.7 ml each of 0.6 and 0.25 M sucrose. The flotation step was conducted at 370,000\( \times \)g for 54 min. and the sarcolemmal vesicles were collected from the 1.0 M/0.6 M sucrose interface. The sucrose was diluted with homogenizing buffer and sarcolemmal fraction concentrated by centrifugation at 100,000\( \times \)g for 30 min. in 20 mM Tris buffer, 0.25 M sucrose, and stored frozen at -20°C for later use.

Ouabain-sensitive Na/K-ATPase activity was measured at 37°C in a spectrophotometric enzymecoupled assay as described.\(^12\) Sarcolemm (\( \sim \)2 \( \mu \)g) was incubated in medium containing (in mmol/L) Tris-HCl 30, KCl 20, MgCl₂ 3, EDTA 1, Phospho(enol)Pyruvate 15, NADH 1, ATP 3, \( ^{3}\)H-ouabain, 10, and 46 units of lactic dehydrogenase and 3.3 units of pyruvate kinase at pH 7.2, with NaCl=0, 2.5, 5, 10, 20, 40, 80 or 120 mM. The decline in NADH absorbance at 340 nm is used to calculate ATPase rate sensitive to 10 mmol/L ouabain.

**Statistical analysis**

Data are expressed as mean±SEM. Statistical discriminations were performed with Student’s t test (paired when appropriate) with p<0.05 considered significant.

**Results**

The reproducibility of repeated activations of Na-pump was tested (Fig S1A). NKA was inhibited in K-free solution, which allowed [Na], to gradually rise to ~45 mM. Then the pump was re-activated by returning [K], to 4 mmol/L (and replacing extracellular NaCl with TEA-Cl). A typical finding was that the rise in [Na], during NKA inhibition was faster in the second run. We do not know why, but this occurred whether or not there was ISO in the second NKA blockade. However, we focused on analysis of NKA function during [Na], decline. The inset in Fig S1A shows that sequential runs in the same cell produced virtually identical [Na], decline. This was confirmed in further analysis in 3 cells (Fig S1B). That is, there was no systematic
A difference in either the $V_{\text{max}}$ or $K_m$ for Na between repeated runs.

References


2. Despa S, Bers DM. Frequency-dependent acceleration of relaxation in the heart depends on CaMKII, but not phospholamban. J Mol Cell Cardiol 2002;34:975-984.


