Expression and Phosphorylation of the Na-Pump Regulatory Subunit Phospholemman in Heart Failure

Julie Bossuyt, Xun Ai, J. Randall Moorman, Steven M. Pogwizd, Donald M. Bers

Abstract—Intracellular [Na] is ~3 mmol/L higher in heart failure (HF; in our arrhythmogenic rabbit model;3), and this can profoundly affect cardiac Ca and contractile function via Na/Ca exchange and Na/H exchange. Na/K-ATPase is the primary mechanism of Na extrusion. We examine here in HF rabbits (and human hearts) expression of Na/K-ATPase isoforms and phospholemman (PLM), a putative Na/K-ATPase regulatory subunit that inhibits pump function and is a major cardiac phosphorylation target. Na/K-ATPase α1- and α2-isofoms were reduced in HF in rabbit ventricular homogenates (by 24%) and isolated myocytes (by 30% and 17%), whereas α3 was increased (50%) in homogenates and decreased (52%) in myocytes (P<0.05). Homogenate Na/K-ATPase activity in left ventricle was also decreased in HF. However, we showed previously that Na/K-ATPase characteristics in intact ventricular myocytes were unaltered in HF. To reconcile these findings, we assessed PLM expression, phosphorylation, and association with Na/K-ATPase. PLM coimmunoprecipitated with Na/K-ATPase α1 and α2 in control and HF rabbit myocytes. PLM expression was reduced in HF by 42% in isolated rabbit left ventricular (LV) myocytes, by 48% in rabbit LV homogenates, and by 24% in human LV homogenate. The fraction of PLM phosphorylated at Ser-68 was increased dramatically in HF. Our results are consistent with a role for PLM analogous to that of phospholamban for SR Ca-ATPase (SERCA): inhibition of Na/K-ATPase function that is relieved on PLM phosphorylation. So reduced Na/K-ATPase expression in HF may be functionally offset by lower inhibition by PLM (because of reduced PLM expression and higher PLM phosphorylation).

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Key Words: heart failure ■ Na/K-ATPase ■ phospholemman ■ FXYD proteins

Cardiac contractile dysfunction and arrhythmias in heart failure (HF) are attributable in part to altered myocyte Ca handling, but altered intracellular [Na] ([Na]i) and Na transporters can contribute via Na/Ca and Na/H exchange.1 Moreover, upregulation of Na/Ca exchange in HF may magnify the functional impact of the interplay between Ca handling and [Na],1-2 and [Na], is elevated in hypertrophy and HF.3-8 Two possible explanations for the higher [Na], are1 decreased Na efflux (eg, via Na/K-ATPase) and2 increased Na influx.

Several biochemical studies have reported decreased expression or altered isoform expression of Na/K-ATPase in HF.4,5,9,10 However, few studies have assessed cellular Na/K-ATPase function during HF, and they do not all agree. Rats with postmyocardial infarction induced HF, had decreased Vmax, and unchanged [Na], affinity.11 In myocytes from dogs with chronic atrioventricular block and hypertrophy, maximal pump current was unaltered, whereas [Na], affinity was slightly reduced.12 We found unaltered myocyte Na/K-ATPase pump characteristics in a nonischemic rabbit HF model, but 3 mmol/L higher [Na].3 The [Na], elevation in HF was attributable largely to increased tetrodotoxin-sensitive Na influx.3 Our first aim here was to determine whether Na/K-ATPase isoform expression was reduced or shifted in our rabbit HF model (as seen in human HF). It was.

Altered regulation of fewer Na/K pumps in HF might reconcile these disparate findings, so we examined the expression and phosphorylation state of the Na/K-ATPase regulatory protein phospholemman (PLM). PLM (or FXYD1) is a member of the FXYD gene family of plasmalemmal proteins that includes the Na/K-ATPase regulatory protein phospholemman (PLM). PLM expression and phosphorylation were altered in HF. In rabbit HF models, Na/K-ATPase expression was decreased, whereas Na/K-ATPase activity was unaltered. However, we showed previously that Na/K-ATPase characteristics in intact ventricular myocytes were unaltered in HF. To reconcile these findings, we assessed PLM expression, phosphorylation, and association with Na/K-ATPase. PLM coimmunoprecipitated with Na/K-ATPase α1 and α2 in control and HF rabbit myocytes. PLM expression was reduced in HF by 42% in isolated rabbit left ventricular (LV) myocytes, by 48% in rabbit LV homogenates, and by 24% in human LV homogenate. The fraction of PLM phosphorylated at Ser-68 was increased dramatically in HF. Our results are consistent with a role for PLM analogous to that of phospholamban for SR Ca-ATPase (SERCA): inhibition of Na/K-ATPase function that is relieved on PLM phosphorylation. So reduced Na/K-ATPase expression in HF may be functionally offset by lower inhibition by PLM (because of reduced PLM expression and higher PLM phosphorylation).
and human HF, we found reduced PLM expression and increased PLM phosphorylation. PLM still associates with Na/K-ATPase α-subunits in HF. These findings are consistent with a role for PLM analogous to that of PLB for SR Ca-ATPase (SERCA). That is, PLM may inhibit Na/K-ATPase function (by reducing [Na+]i sensitivity),\textsuperscript{13,19} with this effect reversed on PLM phosphorylation.\textsuperscript{19} Thus, reduced Na/K-ATPase expression in HF may be functionally offset by the reduced expression and increased phosphorylation of PLM.

### Materials and Methods

**HF Model**

HF was induced in New Zealand White rabbits by combined aortic insufficiency and stenosis as described previously.\textsuperscript{20-23} Rabbits were studied 7.1±1.8 months later, when the end-systolic dimension exceeded 1.20 cm (measured by 2D echocardiography under ketamine sedation).\textsuperscript{22} Myocytes were isolated via Langendorff perfusion as described,\textsuperscript{21} with back-flow across the incompetent aortic valve in HF rabbits blocked by an inflated balloon-tipped catheter. Myocardium pieces were also flash-frozen and stored at -80°C. Protocols were approved by the University of Illinois Chicago (UIC) and Loyola University Chicago institutional animal care and use committees.

**Human Left Ventricular Tissue**

Left ventricular (LV) tissue was obtained from 14 human hearts (8 men, 6 women; ejection fraction 18.6±3.2%) after explantation in end-stage idiopathic dilated cardiomyopathy (n=5) or ischemic cardiomyopathy (n=9) performed at Loyola University Chicago and UIC hospitals. Ten of these patients were taking digoxin, and 10 had implantable defibrillators for documented ventricular tachycardia. Four nonfailing human hearts (which could not be used for transplantation) were obtained from the Regional Organ Bank of Illinois. Protocols were approved by the human studies committees of Loyola and UIC.

**Cell Lysate and Homogenate Preparation**

Isolated myocytes were rinsed and lysed in ice-cold lysis buffer containing (in mmol/L): 150 NaCl, 50 Tris, pH 7.5, 0.5% NP-40 (including phosphatases and kinases) and agonists from Calbiochem, immunoblotting materials were obtained from Bio-Rad, enzymes and protease inhibitor cocktail (PIs; Calbiochem; type 3). Cell lysates were flash-frozen and stored at -80°C. Myocyte lysates were also prepared after cellular treatment with receptor agonists and kinase activators. Alternatively, after cell lysis, protein was phosphorylated with purified PKA and PKC as described previously.\textsuperscript{23} Ventricular homogenates were prepared by grinding frozen tissue in a mortar and Dounce homogenizer for buffer containing (in mmol/L): 140 NaCl, 25 imidazole, and 1 EDTA, pH 7.3. The extract was diluted 1:1 with detergent-free buffer to 1 mg/mL cardiac homogenate (18.6 mg/mL; Upstate), anti-PLM-CP68 antibody.\textsuperscript{24} After incubation with horseradish peroxidase-labeled secondary antibody, blots were developed using enhanced chemiluminescence (Amersham Pharmacia Biotech). Equal protein loading was ensured by reprobing with GAPDH. HF signals were normalized to control sample signals on the same gels.

**Immunoprecipitations**

To immunoprecipitate Na/K-ATPase α-subunit, we used either the pan-specific probe (αs) or isoform-specific antibodies noted above as described previously.\textsuperscript{13,15} To immunoprecipitate PLM, PLM-C2 and PLM-CP68 antibodies described above were used. As controls, we immunoprecipitated SERCA2, plasma membrane Ca-ATPase (PMCA) (Affinity Bioreagents), and nonimmune IgG. Cardiac membranes from homogenates or isolated myocytes were solubilized for 30 minutes at 4°C using 6 mg/mL n-dodecyl octaethylene glycol monoether detergent (C\textsubscript{16}E\textsubscript{6}; Calbiochem) in buffer containing (in mmol/L): 140 NaCl, 25 imidazole, and 1 EDTA, pH 7.3. The extract was diluted 1:1 with detergent-free buffer to 1 mg/mL cardiac protein and centrifuged for 30 minutes at 20 000 × g at 4°C to remove debris (pellet Na/K-ATPase was very low). Supernatant was incubated with primary antibodies or control IgG (1 to 2 μg/mL) overnight at 4°C with end-over-end rotation. Immune complexes were collected after 2 hours of incubation with 40 μL of secondary goat anti-rabbit or goat anti-mouse IgG antibodies covalently bound to agarose beads and centrifugation at 10 000 × g for 10 minutes at 4°C (washed five times with solubilization buffer containing 0.05% C\textsubscript{16}E\textsubscript{6}). Final pellets were resuspended in sample buffer, centrifuged at 10 000 × g for 10 minutes and supernatants used to load onto gels.

**Na/K-ATPase Activity**

Ouabain-sensitive Na/K-ATPase activity was determined using fresh LV homogenate in buffer containing (in mmol/L): 110 NaCl, 50 Tris, 10 Na\textsubscript{2}SO\textsubscript{4}, 1 EDTA, 4 MgCl\textsubscript{2}, 20 KCl, and 3.5 ATP, ±1 ouabain, pH 7.4, at 37°C for 6 minutes (within the linear assay range). The reaction was stopped with fresh phosphate reagent (20 mmol/L Na molybdate, 2% H\textsubscript{2}SO\textsubscript{4}), malachite green (37% solution) was added, and the resulting phospho-molybdate-malachite green complex was quantified by measuring absorbance at 650 nm against a NaHPO\textsubscript{4} standard.\textsuperscript{25}

**Materials and Data Analysis**

Immunoblotting materials were obtained from Bio-Rad, enzymes (including phosphatases and kinases) and agonists from Sigma. Results are representative of at least three separate hearts and expressed as means±SE where quantified (n=number of hearts used). Unpaired Student’s t test was used for comparisons (P<0.05 was considered significant).

### Results

**Depressed Ventricular Function in HF**

HF rabbits exhibited depressed LV systolic function based on increases in LV end-diastolic and end-systolic dimension (by 48% and 67%, respectively [P<0.001]), and decreased LV fraction shortening (by 23%; from 36.2±0.9 to 27.8±1.4; P<0.001) versus baseline recordings in the same animals. Studies in rabbit myocytes were complemented by experiments in human LV tissue from 14 patients with end-stage HF, in whom LV ejection fraction was severely depressed (18.6±3.2%). No differences were seen among LV tissues.
from the different HF hearts based on etiology or the use of digoxin, so data from these 14 failing hearts were pooled.

**Na/K-ATPase Isoform Expression in HF**

Total Na/K-ATPase α-subunit expression in HF rabbits was assessed (Figure 1) using a pan-specific probe (α5), which detects all three α-isofoms equally well. Densitometric analysis revealed a 36% reduction in rabbit HF myocytes (Figure 1A). A similar trend was observed in human HF myocytes (21% reduction; Figure 1A) and LV homogenates (17% reduction; \( P = 0.06 \)). Ouabain-sensitive ATPase activity was also decreased in LV homogenates of HF rabbits (4.88 ± 1.07 versus 2.36 ± 0.71 μmol/mg per hour; Figure 1B). In heart, α1 is the most abundant α-isofom, but relative isoform expression cannot be gleaned from Figure 1.

Figure 2 documents Na/K-ATPase isoform expression in HF rabbits. Using isoform-specific antibodies, all three α-isofoms (α1, α2, and α3) as well as the β1-subunit were detected. Representative blots and densitometric analysis (n = 7) in LV homogenates (Figure 2A) show reduced α1 and α2 (by 24% each) in HF tissue but increased α3-isofom expression (by 50%). Expression of β1-subunit was not significantly altered. Because Na/K-ATPase is a ubiquitous protein, experiments were also performed in lysates from freshly isolated myocytes (Figure 2B) to evaluate changes in ventricular myocytes (versus other cells). Myocyte (n = 14) expression of all three α-isofoms was reduced (by 30%, 17%, and 58% for α1, α2, and α3, respectively), whereas β1 remained unchanged. Thus, in HF rabbits, there is upregulation of α3 in nonmyocytes but downregulation of all three α-isofoms in HF myocytes. This finding emphasizes the importance of studying expression in isolated myocytes (versus homogenates) when myocytes are the cell type of interest, especially for ubiquitous proteins like Na/K-ATPase.

**Detection of PLM and Its Phosphorylation State**

PLM can be phosphorylated by PKA at Ser-68 and by PKC at Ser-63 and Ser-68.26 Two antibodies were used: PLM-CP68 is a Ser-68 phosphorylation specific PLM antibody, whereas PLM-C2 detects PLM with some preference for dephosphorylated PLM (but not absolute).24,27 Figure 3 shows typical results (n ≥ 3) that these antibodies recognize rabbit PLM and its phosphorylation state. Figure 3A shows treatment of myocyte lysates with purified enzymes, whereas Figure 3B shows the influence of agents applied to intact myocytes before cell lysis and homogenization. The PLM-C2 signal is maximal after dephosphorylation (by PP1a and PP2a) and minimal, but still apparent, after phosphorylation with PKA or PKC. The PLM-CP68 signal is undetectable on dephosphorylation and increases in response to phosphorylation by PKA or PKC. In intact myocytes (Figure 3B, control), PLM is partially phosphorylated at Ser-68 in the absence of agonists but can be further increased by activation of PKA stimulation (Iso or forskolin) or PKC (phenylephrine, endothelin-1, and phorbol ester). Curiously, ouabain sometimes increased phospho-PLM (not significantly), a point not further explored. Furthermore, the range of response to treatment suggests that phosphorylation of PLM is likely to change significantly in the cell, and this may alter its function and that of Na/K-ATPase.

**Interaction of PLM With the Na/K-ATPase**

Coimmunoprecipitations were performed to test whether PLM can form stable complexes with Na/K-ATPase.
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**Figure 3.** Detection of PLM expression and phosphorylation. A, Myocyte lysates were exposed to purified kinases and phosphatases and probed with 2 antibodies (PLM-C2 and PLM-CP68) sensitive to phospho-PLM (P-PLM) and dephospho-PLM (deP-PLM). Protein (50 μg) was treated with, respectively, 15 U PKA (±100 nmol/L protein kinase A inhibitor), 0.25 μg PKC, and 2U PP1a plus 0.1 μg PP2a for 20 minutes at 37°C. B, Modulation of PLM phosphorylation by treatment of intact myocytes with 1 mmol/L ouabain, 1 μmol/L iso, 100 mmol/L forskolin, 10 μmol/L phenylephrine (PE), 50 mmol/L endothelin-1 (ET-1), or 100 mmol/L PDBu for 20 minutes at 25°C. After treatments, further phosphorylation changes were stopped by flash-freezing with phosphatase inhibitors in the lysis buffer. All results representative of at least 3 replicates.

**Figure 4.** Immunoprecipitation (IP) of PLM with Na/K-ATPase isoforms. PLM-C2 was used as probe for phospho-PLM (P-PLM) and dephospho-PLM (deP-PLM) as detected by isoform-specific antibodies (only α1 signal is shown in Figure 4A). Note that virtually all the α1-subunit was brought down in the PLM immunoprecipitate (none seen in the supernatant). This is consistent with all α1-subunits being partnered to PLM in situ. Because some PLM is phosphorylated (see CP68 signal in Figure 4A), this may indicate that the C2 antibody can immunoprecipitate phospho-PLM, although it has apparently weaker affinity for this form on Western blots. Figure 4B shows that each α-isoform-specific antibody can also immunoprecipitate PLM. Nonimmune IgG was used as control (mock precipitation) and failed to bring down PLM or the α-isoforms, respectively. Note that the PLM-CP68 antibody detected phosphorylated PLM in this immunoprecipitate (especially in the α2-directed immunoprecipitation). Indeed, the ratio of CP68/C2 signal in the same lanes (indicative of fractional PLM phosphorylation) was significantly higher in immunoprecipitates of α2 versus α1 (1.37±0.32 versus 0.23±0.01; P<0.05; n=3). This may reflect either greater propensity for phosphorylation of PLM that is associated with α2 or a stronger affinity of α2 (versus α1) for phosphorylated PLM.

**Figure 4E** shows that PLM immunoprecipitations pull down virtually all of the Na/K-ATPase α-subunits (assessed with the α5 antibody) in control rabbit myocyte. However, in HF myocytes, PLM immunoprecipitation leaves behind a small but detectable amount of Na, K-ATPase α-subunit (Figure 4E, right). This could either be because of a reduction in PLM–Na/K-ATPase affinity or stoichiometry in HF.

PMCA (sarcolemmal) failed to bring down PLM (Figure 4C and 4D). PMCA could also not be detected in the anti-PLM–mediated precipitation (Figure 4D, right). These results indicate that the association of PLM with the Na/K-ATPase is: (1) specific for Na/K-ATPase versus other P-type ATPases and (2) not an artifact of precipitating other sarcolemmal proteins. Thus, PLM interacts with all Na/K-ATPase α-isoforms in the rabbit heart and is not eliminated by PLM phosphorylation. Similar results were obtained in human cardiac homogenates with respect to lack of Na/K-ATPase α-isoform specificity (data not shown).

**Figure 4D** shows that PLM immunoprecipitations pull down virtually all of the Na/K-ATPase α-subunits (assessed with the α5 antibody) in control rabbit myocyte. However, in HF myocytes, PLM immunoprecipitation leaves behind a small but detectable amount of Na, K-ATPase α-subunit (Figure 4E, right). This could either be because of a reduction in PLM–Na/K-ATPase affinity or stoichiometry in HF.

**PLM Expression and Phosphorylation in HF**

To test the Na/K-ATPase specificity of PLM coimmunoprecipitation, we used related P-type ATPases. Immunoprecipitations with anti-SERCA2 (the SR Ca-ATPase) and anti-α-subunits (and which isoforms) in the rabbit heart (Figure 4). These experiments were performed in isolated myocytes to avoid complications by nonmyocytes in tissue homogenate (although homogenates were used for some human samples). We found that the PLM-C2 antibody could immunoprecipitate all three isoforms (α1, α2, and α3) of the Na/K-ATPase, as detected by isoform-specific antibodies (only α1 signal is shown in Figure 4A). Note that virtually all the α1-subunit was brought down in the PLM immunoprecipitate (none seen in the supernatant). This is consistent with all α1-subunits being partnered to PLM in situ. Because some PLM is phosphorylated (see CP68 signal in Figure 4A), this may indicate that the C2 antibody can immunoprecipitate phospho-PLM, although it has apparently weaker affinity for this form on Western blots. Figure 4B shows that each α-isoform-specific antibody can also immunoprecipitate PLM. Nonimmune IgG was used as control (mock precipitation) and failed to bring down PLM or the α-isoforms, respectively. Note that the PLM-CP68 antibody detected phosphorylated PLM in this immunoprecipitate (especially in the α2-directed immunoprecipitation). Indeed, the ratio of CP68/C2 signal in the same lanes (indicative of fractional PLM phosphorylation) was significantly higher in immunoprecipitates of α2 versus α1 (1.37±0.32 versus 0.23±0.01; P<0.05; n=3). This may reflect either greater propensity for phosphorylation of PLM that is associated with α2 or a stronger affinity of α2 (versus α1) for phosphorylated PLM.

To test the Na/K-ATPase specificity of PLM coimmunoprecipitation, we used related P-type ATPases. Immunoprecipitations with anti-SERCA2 (the SR Ca-ATPase) and anti-
Phoresis to allow uniform (phosphorylation-independent) detection with PLM-C2 antibody (Figure 5A, right). Figure 5B shows that in HF rabbits total PLM expression was reduced in isolated rabbit myocytes (43%) and LV homogenates (48%) and in human LV homogenates (26%). The reduction in PLM expression is somewhat more extensive than for total \( \alpha \)-\( \alpha \)-expression levels (ie, \( \approx 50\% \) for PLM versus 24% to 36% for \( \alpha \)). This is consistent with reduced PLM/Na/K-ATPase ratio in HF and so less inhibitory effect of PLM on some pumps (although absolute stoichiometry is unknown).

Figure 5C shows that without intentional PLM dephosphorylation (Figure 5A, left), there is more phospho-PLM (CP68) in HF, despite the much lower PLM expression level. This indicates that a much higher fraction of PLM is phosphorylated in HF. Complementary data were seen with the PLM-C2 signal in Figure 5C (de-P-PLM), which prefers de-phospho-PLM. The C2-PLM signal was reduced by a greater extent than the downregulation of PLM expression, again consistent with a greater fraction of PLM being phosphorylated in HF. Because the C2-PLM antibody has some affinity for phospho-PLM (Figure 3A), we assign more quantitative credence to the PLM-CP68 data (which show no signal for deP-PLM). Normalizing the P-PLM data in Figure 5C to the PLM expression data in Figure 5B, the fraction of PLM phosphorylation is about doubled (Figure 5B; \%P-PLM). A similar trend was seen in rabbit and human LV homogenate. In human HF, the P-PLM/PLM ratio was 162% of control (versus the 217% in rabbit HF myocytes in Figure 5C).

PLM-Na/K-ATPase association in HF was assessed by immunoprecipitation with PLM-C2 and PLM-CP68 antibodies (Figure 6). Even in HF, in which PLM is less abundant and more PLM is phosphorylated, the PLM-C2 antibody still immunoprecipitates Na/K-ATPase \( \alpha \) (although total PLM and associated Na/K-ATPase \( \alpha \)-subunit in HF was \( \approx 50\% \) of control; Figure 6A; \( n=4 \); \( P<0.05 \)). Furthermore, all three \( \alpha \)-isoforms could still be detected in the PLM precipitate from rabbit and human HF (Figure 6B). The phospho-PLM antibody (CP-68) immunoprecipitates less PLM (ie, only P-PLM; data not shown). Nevertheless, Figure 6C shows that CP68 immunoprecipitates more P-PLM (and \( \alpha \)) in HF versus control rabbit myocytes (by 40%; \( n=3 \); \( P<0.05 \)), consistent with greater P-PLM in HF (Figure 5) and the phosphospecific nature of the antibody. We conclude that in HF, total PLM expression is reduced, but more PLM is phosphorylated.

Figure 5. PLM expression and phosphorylation. Example immunoblots (A) and pooled data (B) of PLM expression in control (Ctl) and HF in rabbit and human. Total PLM was determined as PLM-C2 signal after complete dephosphorylation with PP1a and PP2a treatment (see Methods). Signals are normalized to control (\( *P<0.05 \); \( n=8 \) and 7 for rabbit myocytes and homogenates, and \( n=3 \) and 10 for human nonfailing and HF). C, Enhanced PLM phosphorylation in HF rabbit myocytes. PLM-CP68 and PLM-C2 signals were obtained without previous dephosphorylation, and HF signals are normalized to parallel control (\( \cdot P<0.05 \); \( n=8 \)). Relative phosho-PLM was determined as PLM-CP68 signal/total PLM-C2 signal.

Figure 6. PLM still interacts with Na/K-ATPase in HF. A, IP with PLM-C2 (after dephosphorylation) probed with pan-specific Na/K-ATPase probe \( \alpha 5 \) (\( n=4 \) for control [Ctl] and HF). B, IP with PLM-C2, probed with isoform-specific antibodies for Na/K-ATPase (\( n=3 \)). C, IP with PLM-CP68 probed with \( \alpha 5 \) and PLM-C2 (\( n=3 \)).
Discussion

In our rabbit HF model, [Na], is  3 mmol/L higher than control (at rest and during stimulation), similar to other hypertrophy and HF models, including human. This 3 mmol/L [Na], change may seem small, but it has tremendous impact on Ca regulation via Na/Ca exchange. 

Several studies have indicated reduced Na/K-ATPase expression in hypertrophy or HF. Thus, we expected the reduced Na/K-ATPase expression in our rabbit (and human) HF measurements. Although this was the case, our previous work showed elevated [Na], despite cellular Na/K pump V max and [Na], dependence that was essentially unchanged in HF. 

This suggested that there is either: (1) a higher fraction of Na/K-ATPase molecules on the cell surface in HF (and Na/K-ATPase is known to be partially internalized in other cell types), or (2) altered Na/K-ATPase regulation in HF. Our results here are consistent with the latter possibility (but do not rule out the former).

Na/K-ATPase Expression in HF

The Na/K-ATPase has three α-subunit isoforms (α1, α2, and α3) that differ in affinity for Na and ouabain, and several β-subunit isoforms (although heart expresses mainly β1). So isoform shifts could affect Na/K-ATPase activity in HF. In human HF, Schwinger et al reported downregulation of Na/K-ATPase α1, α3, and β1 but not α2. In our rabbit HF myocytes, all three isoforms were reduced (although α2 did not reach significance; Figure 2B). The fact that α3 was increased in LV homogenate but reduced in isolated myocytes suggests a marked upregulation of α3 expression in nonmyocyte cells in the heart. Using an antibody that is expected to detect all three isoforms equally, we conclude that the overall downregulation of Na/K-ATPase in rabbit HF myocytes is by  36% and in human HF by  17%.

Not all data on Na/K-ATPase isoform expression agree in animal models of hypertrophy and HF. However, most find reduced Na/K-ATPase α-subunit expression or 1H-ouabain binding sites, especially in human HF. Part of the disagreement reported may reflect differences in species, HF models, and differences in human HF patient population. More important, because virtually all of the studies were performed in tissue homogenates, some discrepancies may also be attributable to nonmyocyte contributions, as our results in Figure 2 indicate. Notably, several studies show upregulation of α3 in rat hypertrophy or HF on the basis of tissue not myocyte measurements.

PLM as a Na/K-ATPase Modulator

We considered PLM changes and Na/K-ATPase regulation in explaining reduced Na/K-ATPase expression with unaltered cellular function. That is, there may be fewer pumps in HF, but those could be more active because of altered PLM regulation. PLM is FXYD1, the first member of the FXYD family of seven single-membrane–spanning mammalian proteins. These proteins have been identified recently as tissue-specific regulators of Na/K-ATPase, and other members include the Na/K-ATPase γ-subunit (FXYD2), the regulator of renal Na/K-ATPase (FXYD4 or corticosteroid hormone–induced factor), and the related PLM-like shark rectal gland protein. PLM is highly expressed in heart (and brain) and is unique among mammalian FXYD proteins in having demonstrable phosphorylation sites at the cytosolic carboxyl terminus. PLM phosphorylation sites are major targets of PKA and PKC in heart, but their function has been unclear.

Crambert et al expressed PLM and Na/K-ATPase α1- and α2-subunits in Xenopus oocytes and demonstrated that PLM reduced the apparent affinity for [Na], and, to a lesser extent, for [K,]. Our recent data in PLM knockout mice indicate that the PKA-dependent stimulation of Na/K-ATPase activity in ventricular myocytes is attributable to PLM phosphorylation. The apparent [Na], affinity of Na/K-ATPase in PLM knockout myocytes was higher than in wild-type mice (extending oocytes studies above) but was the same in knockout as in wild-type mice after maximal PKA activation. This is remarkably parallel to how SR Ca-ATPase is inhibited by PLB and how phosphorylation relieves that inhibition.

Here, we found specific association of PLM with rabbit ventricular myocyte Na/K-ATPase (for α1, α2, and α3) in control and HF conditions, and even when PLM is phosphorylated (Figures 4 and 6). Fuller et al also found the PLM–α1 association to be unaffected by PKA-dependent phosphorylation. Crambert et al first showed that PLM associates with α1 and α2 Na/K-ATPase isoforms and that the association is more robust for α-β1 than α-β2 isoforms in an oocyte transfection system. In the cerebellum, the interaction appears to be nonisofrom specific. Shatton et al showed that PLM associates with α1- but not α2-subunits in rat and guinea pig myocytes (using immunoprecipitation and immunofluorescence). This difference may be attributable to isoform expression patterns in these species but may also reflect relative stability of the interaction under different immunoprecipitation conditions.

Our methods here were like those described by Crambert et al in terms of detergent and ionic conditions. Under these conditions, we do find the PLM–α-subunit interaction to be specific for Na/K-ATPase (because neither sarcolemmal or SR Ca-pumps were coimmunoprecipitated). However, if we use more aggressive detergent conditions, we and Crambert et al could disrupt the PLM–α2 association (and PLM–α3 association in our case) but could not disrupt the coimmunoprecipitation of PLM with α1. We conclude that PLM can interact with all three isoforms, but that the affinity of PLM for the α1-isoform is the highest.

Altered Na/K-ATPase and PLM in HF

This is the first report on PLM expression and phosphorylation in HF in our rabbit HF model and human HF. We find Na/K-ATPase expression is reduced but that PLM expression is even more greatly reduced, indicating lower PLM:Na/K-ATPase stoichiometry (Figure 1A versus Figures 5B and 4E). This could result in less overall Na/K-ATPase inhibition by PLM. The combination of reduced amount and more phosphorylated PLM may result in fewer Na/K-ATPase molecules.

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producing relatively normal Na extrusion. Despa et al used physiological extracellular [K] (4 mmol/L), not saturating with respect to \( V_{\text{max}} \), and PLM can also inhibit Na/K-ATPase K affinity. Thus, a modest decrease in true biochemical \( V_{\text{max}} \) could have been missed by Despa et al. Our results in human HF were similar to rabbit HF, for Na/K-ATPase and PLM (albeit to a lesser extent).

Thus, downregulation of Na/K-ATPase expression in HF may be functionally offset by less PLM-dependent inhibition. Which comes first is unknown. However, the relative normalcy of Na/K-ATPase function in HF myocytes could imply that one of these is compensatory to normalize the Na extrusion capability of the myocyte.

Different models of hypothermia or HF may vary in PLM alterations. Short-term postmyocardial infarction rat hearts show upregulation of PLM mRNA. Altered PLM expression may also modify cardiac Ca transients, which would be an expected consequence of altered Na/K-ATPase function and Ca shifts via Na/Ca exchange. Altered myocyte Ca transients were indeed reported when PLM was overexpressed or downregulated in cultured adult rat ventricular myocytes. In addition to indirect Ca transport changes secondary to altered Na/K-ATPase function, those authors suggest that PLM may also directly modulate Na/Ca exchange function.

In conclusion, we found reduced Na/K-ATPase expression in this nonischemic, arrhythmogenic rabbit HF model and human HF, and reduced expression and enhanced phosphorylation of PLM. Because dephosphorylated PLM is an important endogenous Na/K-ATPase inhibitor, this may explain the relatively unaltered cellular Na/K-ATPase function in HF. The dynamic ability of PLM and its phosphorylation state to modulate [Na], and the strong dependence of Ca regulation in heart via Na/Ca exchange make it important to understand how PLM regulates Na/K-ATPase. This is especially true in HF, in which Na/Ca exchange is upregulated and has been implicated in systolic dysfunction and arrhythmogenesis.

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References


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