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Phospholemman: a novel cardiac stress protein.

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Abstract

Phospholemman (PLM), a member of the FXYD family of regulators of ion transport, is a major sarcolemmal substrate for protein kinases A and C in cardiac and skeletal muscle. In the heart, PLM co-localizes and co-immunoprecipitates with Na^+-K^+-ATPase, Na^+/Ca^{2+} exchanger and L-type Ca^{2+} channel. Functionally, when phosphorylated at serine^{68}, PLM stimulates Na^+-K^+-ATPase but inhibits Na^+/Ca^{2+} exchanger in cardiac myocytes. In heterologous expression systems, PLM modulates the gating of cardiac L-type Ca^{2+} channel. Therefore, PLM occupies a key modulatory role in intracellular Na^+ and Ca^{2+} homeostasis and is intimately involved in regulation of excitation-contraction (EC) coupling. Genetic ablation of PLM results in a slight increase in baseline cardiac contractility and prolongation of action potential duration. When hearts are subjected to catecholamine stress, PLM minimizes the risks of arrhythmogenesis by reducing Na^+ overload and simultaneously preserves inotropy by inhibiting Na^+/Ca^{2+} exchanger. In heart failure, both expression and phosphorylation state of PLM are altered and may partly account for abnormalities in EC coupling. The unique role of PLM in regulation of Na^+-K^+-ATPase, Na^+/Ca^{2+} exchanger and potentially L-type Ca^{2+} channel in the heart, together with the

changes in its expression and phosphorylation in heart failure, make PLM a rational and novel target for development of drugs in our armamentarium against heart failure.

Introduction

Phospholemman (PLM) was initially identified by Larry Jones in 1985 (58) as a 15-kDa sarcolemmal (SL) protein that is phosphorylated in response to isoproterenol and is distinct from phospholamban (PLB)(58). Follow-up studies indicated that this 15-kDa SL protein is also phosphorylated by protein kinase (PK) C (59) and α-adrenergic agonists (38). In 1991, this 15-kDa SL phosphoprotein was purified, the complete protein sequence determined by Edman degradation, the cDNA cloned, and the name "phospholemman" was coined (53). In 1997, the human PLM gene is localized to chromosome 19q13.1 (11).

PLM is synthesized as a 92 amino acid peptide containing at its N-terminus a 20 amino acid signal peptide which is cleaved off during processing. The mature protein contains 72 amino acid residues with a calculated molecular weight of 8409, but a mobility of ~15 kDa in SDS-PAGE gels. The first 17 amino acid residues lie in the extracellular domain. The transmembrane (TM) region contains 20 amino acids (residues 18-37) while the remaining 35 amino acids (residues 38-72) at the C-terminus are in the cytoplasm. Palmer et al. (53) also noted sequence homology between PLM and γ-subunit of Na^+-K^+-ATPase, as well as a short region of sequence

similarity between PLM (at its C-terminus facing the cytoplasm) and PLB (at its N-terminus also facing the cytoplasm). This region of sequence similarity (RSSIRRLST^{69} in PLM and RSAIRRAST^{17} in PLB) contains serines and threonines that are potential phosphorylation sites. Indeed, serine^{68} in PLM and serine^{16} in PLB are phosphorylated by PKA (67, 79).

The extracellular N-terminus of PLM contains a FXYD motif, and the cytoplasmic tail of dog, human and rat PLM contains 3 serines (at residues 62, 63 and 68) and 1 threonine (at residue 69) but threonine^{69} is replaced by serine in mouse PLM. By nuclear magnetic resonance (NMR) (23) and infrared spectroscopy (2), the TM domain of PLM reconstituted in liposomes is an α-helix with a maximum tilt of 15-17^0. Specifically, NMR spectroscopic studies of highly purified PLM in model micelles indicate that the molecule consists of 4 α-helices: H1 (residues 12-17) is in the extracellular N-terminus, H2 (residues 22-38) is the main TM helix followed by the short H3 (residues 39-45), and H4 (residues 60-68) in the C-terminus is connected to H3 by a flexible linker (Fig. 1)(76). In vivo, PKA phosphorylates serine^{68} while PKC phosphorylates serine^{63} and serine^{68} of PLM (79). In vitro studies using PLM fragments suggest that PKA also phosphorylates serine^{63} while PKC phosphorylates threonine^{69} (25). In adult rat myocytes, ~46% of serine^{68} and ~16% of serine^{63} are estimated to be phosphorylated in the resting state (69). Using phospho-specific anti-PLM antibodies (25, 62), ~30-40% of PLM in adult rat myocytes (25, 89) and ~25% of PLM in guinea pig myocytes (66) are phosphorylated under basal conditions. In transfected HEK293 cells, ~30-45 % of exogenous PLM is phosphorylated under resting conditions (92).

Based on observations on Xenopus oocytes in which PLM is overexpressed, Randall Moorman suggested that PLM is a hyperpolarization-activated anion-selective channel (46). When reconstituted in lipid bilayers, PLM forms a channel that is highly selective for taurine

(12) and is thought to be involved in regulation of cell volume in non-cardiac tissues (15, 47). The function of PLM in the heart remains unknown until the dawning of the 21st century.

Phospholemman: Founding Member of the FXYD Family of Regulators of Ion Transport

In 2000, Kathy Sweadner described the FXYD family of regulators of ion transport (73), of which PLM is the first cloned member (FXYD1). At present, there are at least 12 known FXYD proteins, including γ-subunit of Na^+-K^+-ATPase (FXYD2), mammary associated tumor 8 kDa (MAT-8 or FXYD3), channel inducing factor (CHIF or FXYD4), dysadherin (FXYD5; also known as related to ion channel RIC), phosphohippolin (FXYD6), FXYD7, and PLM-S (FXYD10; the shark homolog of PLM). As a family, FXYD proteins are found predominantly in tissues involved in solute and fluid transport (kidney, colon, pancreas, mammary gland, liver, lung, prostate and placenta) or are electrically excitable (heart, skeletal and neural tissues). All FXYD members have the signature FXYD motif in the N-terminus and a single TM domain. Except for γ-subunit of Na^+-K^+-ATPase, all other known members of the FXYD gene family has at least one serine or threonine within the cytoplasmic tail, indicating potential phosphorylation sites. PLM is unique among FXYD proteins in that it has consensus sequence for phosphorylation by PKA (RRXS), PKC (RXXSXR) and never-in-mitosis aspergillus (NIMA) kinase (FRXS/T). PLM is also a substrate for myotonic dystrophy protein kinase (48). The γ-subunit of Na^+-K^+-ATPase is the only member in the FXYD family boasting two alternative splice variants (FXYD2a and FXYD2b).

Phospholemman: Regulator of Cardiac Na^+-K^+-ATPase

In 2002, Kaethi Geering demonstrated that PLM co-immunoprecipitates with α-subunits of Na^+-K^+-ATPase in bovine sarcolemma (14). In isolated adult rat cardiac myocytes, PLM co-localizes with α-subunits of Na^+-K^+-ATPase (Fig. 2). When co-expressed with α- and β-subunits

of Na^+-K^+-ATPase in Xenopus oocytes, PLM modulates Na^+-K^+-ATPase activity primarily by decreasing K_m for Na^+ and K^+ without affecting V_{max} (14). Data obtained from cardiac myocytes or homogenates indicate that PLM inhibits Na^+-K^+-ATPase by reducing its apparent affinities for intracellular Na^+ (9, 17) and external K^+ (29) or decreasing V_{max} (3, 24, 25, 28, 55, 66, 70, 80, 89). When phosphorylated at serine[68], PLM relieves its inhibition on Na^+-K^+-ATPase by decreasing its apparent K_m for Na^+ (17, 80) but not for K^+ (29) and increasing V_{max} (24, 66, 80) .

In terms of molecular interactions between PLM and Na^+-K^+-ATPase, mutational analysis suggests that FXYD proteins (FXYD2, 4 and 7) interact with TM9 segment of Na^+-K^+-ATPase (36). Co-immunoprecipitation and covalent cross-linking studies demonstrate the TM segment of PLM is close to TM2 segment of Na^+-K^+-ATPase (39). Molecular modeling based on Ca^{2+}-ATPase crystal structure in the E_1ATP-bound conformation suggests that the single TM segment of FXYD proteins docks into the groove between TM segments 2, 6 and 9 of the α-subunit of Na^+-K^+-ATPase (39). High resolution crystal structure (2.4 Angstrom) of shark rectal gland Na^+-K^+-ATPase in the $E2.2K^+.P_i$ state indicates that FXYD proteins interact almost exclusively with the outside of TM9 of the α-subunit (65). The role of the signature FXYD(Y) motif is to stabilize interactions between α- and β-subunits of Na^+-K^+-ATPase and residue D (in the FXYD motif) caps the helix and defines the membrane border (65). In transfected HEK293 cells, PLM interacts with either α1- or α2-subunit of Na^+-K^+-ATPase in a 1:1 stoichiometry (9). Phosphorylation of PLM-S causes it to dissociate from shark Na^+-K^+-ATPase (42). However, phosphorylation of PLM did not cause it to dissociate from the α-subunit of Na^+-K^+-ATPase (9, 66). Despite NMR studies of PLM in micelles showing no major conformational changes on phosphorylation of serine[68] (77), in intact cells examined with fluorescence resonance energy

transfer (FRET), interaction between PLM and Na^+-K^+-ATPase is decreased on phosphorylation of PLM (9, 10).

There are 4 isoforms of the catalytic α-subunits of Na^+-K^+-ATPase and expression of a particular α-isoform is both tissue- and species-dependent (7). Human (43, 85) and rabbit (8) hearts are known to express α1-, α2- and α-3 isoforms while rodent hearts express only α1- (ouabain-resistant) and α2-isoforms of Na^+-K^+-ATPase (4, 66, 72, 80). In both adult rat and mouse ventricles, the ouabain-sensitive α2-subunit is preferentially localized to the t-tubules (4, 74) and its activity represents <25% of total Na^+-K^+-ATPase activities (4, 72, 80). PLM co-immunoprecipitates all 3 α-subunits of Na^+-K^+-ATPase in human and rabbit (8), α1- and α2-subunits in mouse (80) and bovine (14), but only α1-subunit in rat (24) and guinea pig (66) hearts. In both wild-type (WT) mouse (80) and guinea pig (66) ventricular myocytes, PLM regulates the activity of α1- but not α2-isoform of Na^+-K^+-ATPase. This conclusion must be tempered with the recent finding that in "SWAP" mouse (21) in which the ouabain affinities of the α-subunits are reversed, PLM regulates the apparent affinities for Na^+ of both α1- and α2-subunitis of Na^+-K^+-ATPase (9). Together with observations made on Xenopus oocytes heterologously expressing PLM and Na^+-K^+-ATPase (6, 14), it is likely the PLM regulates both α1- and α2-subunits of Na^+-K^+-ATPase.

Cardiac Excitation-Contraction Coupling

Both α1- (21) and α2-subunits (74) of Na^+-K^+-ATPase have been implicated in the control of cardiac contractility. Therefore, modulation of Na^+-K^+-ATPase activity suggests an important role for PLM in regulation of inotropy. We will give a brief overview of cardiac excitation-contraction coupling (Fig. 3) which has previously been reviewed by Don Bers in detail (5). During the upstroke of the action potential, Na^+ enters via Na^+ channels and further

depolarizes the sarcolemma. Depolarization activates the voltage-dependent L-type Ca^{2+} channels, allowing extracellular Ca^{2+} to enter as an inward current (I_{Ca}), which contributes to the plateau phase of the action potential. Some Ca^{2+} also enters via the Na^+/Ca^{2+} exchanger (NCX1) operating in the reverse mode (3 Na^+ out: 1 Ca^{2+} in) during this phase of the action potential, although the amount and duration of Ca^{2+} influx via reverse Na^+/Ca^{2+} exchange vary among species. Ca^{2+} entry triggers release of ~2/3 of Ca^{2+} stored in the sarcoplasmic reticulum (SR) via the ryanodine receptor (RyR2). The combination of Ca^{2+} influx and SR Ca^{2+} release abruptly raises the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), allowing Ca^{2+} to bind to troponin C and activate the contractile apparatus. Relaxation requires termination of SR Ca^{2+} release, and $[Ca^{2+}]_i$ to decline so that Ca^{2+} can dissociate from troponin C. About 70-92% of myoplasmic Ca^{2+} is re-sequestered into the SR by sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA2) which is under the control of PLB. To maintain steady-state Ca^{2+} balance, Ca^{2+} that has entered during systole is mainly extruded by NCX1 operating in the forward mode (3 Na^+ in: 1 Ca^{2+} out), with the sarcolemmal Ca^{2+}-ATPase playing a smaller role. Likewise, the small amount of Na^+ that has entered during depolarization is extruded by Na^+-K^+-ATPase during diastole. In this way, the cardiac myocyte maintains beat-to-beat Ca^{2+} and Na^+ balance. Outward K^+ currents contribute to repolarization phase of the action potential.

Among the many transporters and ion channels involved in cardiac Ca^{2+} fluxes, NCX1 is unique in that during an action potential, it participates in Ca^{2+} influx, $[Ca^{2+}]_i$ transient buffering and Ca^{2+} efflux (5). The direction of Ca^{2+} flux (in or out) depends on the thermodynamic driving force determined by the membrane potential (E_m) and the concentrations of Na^+ and Ca^{2+} ions sensed by NCX1. Other than the determinants of its thermodynamic driving force, remarkably little is known about the regulation of NCX1.

In the heart, inhibition of Na^+-K^+-ATPase by PLM is expected to raise intracellular Na^+ concentration ($[Na^+]_i$), thereby decreasing the thermodynamic driving force for forward Na^+/Ca^{2+} exchange (Ca^{2+} efflux) and increasing the driving force for reverse Na^+/Ca^{2+} exchange (Ca^{2+} influx). Both these actions are expected to increase $[Ca^{2+}]_i$ and SR Ca^{2+} load, thereby enhancing cardiac contractility. Indeed, inhibition of Na^+-K^+-ATPase with secondary effects on NCX1 has long been proposed to be the mechanism of positive inotropy of digitalis glycosides (27).

Phospholemman: First Endogenous Regulator of Cardiac Na^+/Ca^{2+} Exchanger

When PLM is overexpressed (1.4 to 3.5-fold) in adult rat left ventricular (LV) myocytes by adenovirus-mediated gene transfer (68, 89, 90), expression of SERCA2, α1- and α2-subunits of Na^+-K^+-ATPase, NCX1 and calsequestrin remains unchanged. As expected, Na^+-K^+-ATPase current (I_{pump}) is decreased in rat myocytes overexpressing PLM, primarily as a result of decrease in V_{max} rather than changes in apparent K_m for Na^+ and K^+ (89). A totally unexpected finding is that both contraction and $[Ca^{2+}]_i$ transient amplitudes (5.0 mM $[Ca^{2+}]_o$, 1 Hz, 37^0C) in myocytes overexpressing PLM are lower, rather than higher, when compared to control rat myocytes overexpressing green fluorescent protein (GFP)(68). This serendipitous but critical observation is inconsistent with the theoretical prediction that inhibition of Na^+-K^+-ATPase by PLM leads to enhanced cardiac contractility. Because the contractile phenotype of myocytes overexpressing PLM is similar to that observed in myocytes in which NCX1 is downregulated (75), and opposite to that in which NCX1 is overexpressed (91), we were the first to propose in 2002 that PLM directly regulates NCX1 activity, independent of its effects on Na^+-K^+-ATPase (68). Follow-up studies in adult rat myocytes demonstrate that PLM co-localizes with NCX1 to the sarcolemma and t-tubules (90), that PLM co-immunoprecipitates with NCX1 (1, 45), that overexpression of PLM inhibits Na^+/Ca^{2+} exchange current (I_{NaCa})(69, 90), and that downregulation of PLM by

antisense increases I_{NaCa} (45). Using HEK293 cells that are devoid of NCX1 and PLM, we demonstrated that cells transfected with NCX1 display the characteristic I_{NaCa}, and that cells co-transfected with PLM demonstrate inhibition of I_{NaCa} as well as Na^+-dependent Ca^{2+} uptake (1). In addition, PLM co-immunoprecipitates with NCX1 in transfected HEK293 cells (1), pig sarcolemmal vesicles (1) and guinea pig ventricular myocyte membranes (82). In cardiac myocytes isolated from PLM-null mice, I_{NaCa} was higher in PLM-null myocytes (87) despite no differences in NCX1 protein levels (78). The cumulative evidence obtained from 3 model systems: adult rat myocytes, HEK293 cells and PLM-null mice are all consistent with our hypothesis that PLM directly regulates NCX1 (13).

NCX1 is a 938 amino acid (939 amino acid in the rat) peptide consisting of an extracellular N-terminal domain comprising the 1^{st} 5 TM segments, a large intracellular loop (residues 218-764), and an intracellular C-terminal domain consisting of the last 4 TM segments (52, 56). The α-repeats of TM segments 2, 3 and 7 of NCX1 are important in ion transport activity (31, 51) while the large intracellular loop contains the regulatory domains of the exchanger (35, 37, 40). Using glutathione S-transferase (GST) pulldown assay, we demonstrated that neither the N- nor the C-terminal TM domains of NCX1 associates with PLM (81). Rather, the cytoplasmic tail of PLM both physically and functionally interacts with the intracellular loop (residues 218-358) of NCX1 (81). Using overlapping NCX1 loop deletion mutants, we further showed that PLM interacts with NCX1 at 2 distinct regions encompassing residues 238-270 and 300-328 (92).

There are significant differences between the mechanisms by which PLM regulates the activities of NCX1 and Na^+-K^+-ATPase. First, phosphorylation of PLM at $serine^{68}$ relieves its inhibition of Na^+-K^+-ATPase (17, 24, 66). By contrast, PLM phosphorylated at $serine^{68}$ is the

active species that inhibits NCX1 (69, 87). Second, the TM segment of FXYD proteins (and by inference PLM) interacts with TM segments 2, 6 and 9 of α-subunit of Na^+-K^+-ATPase (36, 39, 65). By contrast, TM43, a PLM mutant with its cytoplasmic tail truncated, targets correctly to the sarcolemma (69) but does not co-immunoprecipitate NCX1 (81) and has no effect on myocyte contractility (69), suggesting little-to-no association between the TM domains of PLM and NCX1.

Phospholemman: Regulator of Cardiac L-type Calcium Channel

In guinea pig cardiac myocytes, Blaise Peterson recently demonstrated that PLM co-immunoprecipitates not only NCX1 but also L-type Ca^{2+} channels ($Ca_v1.2$)(82). In transfected HEK293 cells and using Ba^{2+} as charge carrier, PLM modulates gating kinetics of $Ca_v1.2$ but not $Ca_v2.1$ (P/Q-type) or $Ca_v2.2$ (N-type) Ca^{2+} channels (82). Specifically, PLM was found to modulate 4 important gating processes of $Ca_v1.2$ channels: (i) activation kinetics were slowed at voltages near the threshold for channel activation; (ii) deactivation kinetics were slowed following voltage steps mimicking human cardiac action potential; (iii) voltage-dependent inactivation was enhanced at voltages corresponding to the plateau phase of the cardiac action potential; and (iv) increased number of channels enter a deep inactivated state from which recovery is slow. When a human cardiac action potential is imposed on HEK293 cells transfected with $Ca_v1.2$ channels, PLM increases Ca^{2+} influx during the repolarization phase of the cardiac action potential (82). The role of PLM phosphorylation in the regulation of $Ca_v1.2$ gating kinetics remains to be elucidated.

The possibility that in cardiac myocytes, PLM may potentially modulate gating kinetics of L-type Ca^{2+} channels, in addition to its known effects on Na^+-K^+-ATPase and Na^+/Ca^{2+} exchanger, renders the interpretation of the effects of PLM expression/phosphorylation on

cardiac contractility extremely complex. However, heterologous expression systems often do not reproduce a protein's native milieu and may distort the stoichiometry of interaction between proteins. A good example is the potentiation of I_{Ca} by adrenergic agonists, so readily observed in cardiac myocytes (63, 88), has yet to be reproduced in heterologous expression systems (34). In addition, using Ca^{2+} as charge carrier, we did not detect any differences in maximal I_{Ca} amplitude, fast and slow inactivation time constants, slope conductance and test potential at which maximal I_{Ca} occurs between WT and PLM-null myocytes (78). Therefore, the physiological significance of regulation of $Ca_v1.2$ by PLM, while intriguing, remains to be established in cardiac myocytes.

Regulation of Single Myocyte Contraction by Phospholemman: Na^+-K^+-ATPase vs. NCX1

In cultured myocytes isolated from PLM-null mice and expressing the phosphomimetic PLM S68E mutant, I_{NaCa} but not I_{pump} is inhibited (70). This is associated with decreased $[Ca^{2+}]_i$ transient and contraction amplitudes (1 Hz, 37^0C) measured at 5.0 but not at 1.8 mM $[Ca^{2+}]_o$: the phenotype that we observed when NCX1 is downregulated (75) or when PLM is overexpressed (68). By contrast, when cultured PLM-null myocytes overexpress the non-phosphorylable PLM S68A mutant, I_{pump} but not I_{NaCa} is inhibited (70). This is associated with no changes in $[Ca^{2+}]_i$ transient and contraction amplitudes at both $[Ca^{2+}]_o$. Therefore, under conditions in which $[Ca^{2+}]_o$ is varied to manipulate the thermodynamic driving force for NCX1, regulation of single cardiac myocyte contractility by PLM is mediated by its inhibitory effects on NCX1 rather than Na^+-K^+-ATPase.

When myocytes are subjected to rapid pacing (2 Hz) and isoproterenol (1 μM) stimulation, $[Na^+]_i$ initially increases but then starts to decline in WT but not in PLM-null myocytes (18, 80). $[Ca^{2+}]_i$ transient and contraction amplitudes follow the time course of $[Na^+]_i$:

initially increase followed by decline in WT but not PLM-null myocytes. When pacing was slowed to 0.5 Hz to minimize the steep rise in $[Na^+]_i$, both $[Ca^{2+}]_i$ transient and contraction amplitudes increase to a lower steady-state level without any time-dependent decline in both WT and PLM-null myocytes (80). These observations suggest that under conditions of high $[Na^+]_i$, phosphorylated PLM activates Na^+-K^+-ATPase to limit intracellular Na^+ overload at the expense of reduced inotropy. Therefore, at the level of a single myocyte, PLM can be shown to regulate Na^+ and Ca^{2+} fluxes (and hence $[Ca^{2+}]_i$ transients and contractility), by either NCX1 or Na^+-K^+-ATPase, depending on experimental manipulations.

Regulation of In Vivo Contractility by Phospholemman: Studies with PLM-null Mice

In 2005, Amy Tucker made a major contribution to the understanding of PLM and cardiac function by successfully engineering the PLM-null mouse (32). There is mild cardiac hypertrophy (3, 32, 80), at least partly due to increased fibrosis in PLM-null hearts (80) since neither LV myocyte length and width (78) nor whole cell membrane capacitance (a measure of cell surface area)(17, 78) is different between WT and PLM-null myocytes. There are no differences in protein levels of α1-, α2-, β1- and β2-subunits of Na^+-K^+-ATPase, SERCA2, PLB, NCX1 and calsequestrin between wild-type and congenic PLM-null hearts (3, 78). The majority of proteins that are differentially expressed between WT and PLM-null hearts are involved in cell metabolism (3). Na^+-K^+-ATPase enzymatic activity (3), I_{pump} (17, 70) and I_{NaCa} (70, 87) are higher in PLM-null hearts, as expected from the relief of inhibition of Na^+-K^+-ATPase and NCX1. There are no changes in I_{Ca} amplitudes but action potential duration is prolonged in PLM-null myocytes (78).

The effects of PLM on cardiac contractility in vivo are complicated and controversial. Using magnetic resonance imaging, Amy Tucker (32) initially reported cardiac hypertrophy and

increased ejection fraction in PLM-null hearts of mice with mixed genetic background (C57BL/6 and 129/SvJ). By contrast, in vivo hemodynamic measurements made by Mike Shattock with conductance catheter introduced by LV puncture in open-chest mice demonstrate no significant differences in measured cardiac indices between WT and PLM-null mice of congenic (C57BL/6) background (3). Our own data in closed-chest catheterized mice show slightly increased baseline +dP/dt in congenic PLM-null hearts (80). Different genetic backgrounds, non-invasive imaging vs. invasive catheterization, different anesthesia, blood loss associated with opening the chest and LV puncture, and heat dissipation in an open-chest mice, may account for these discrepancies. The weight of current evidence, however, indicates that PLM-null hearts contract just as well, if not better, than WT hearts. This is inconsistent with the expectation that with relief of inhibition of Na^+-K^+-ATPase, PLM-null hearts should exhibit lower contractility when compared to WT hearts.

Phospholemman: a Novel Cardiac Stress Protein

When PLM is overexpressed in adult rat LV myocytes, contractility and $[Ca^{2+}]_i$ transient amplitudes measured under physiological conditions (1.8 mM $[Ca^{2+}]_o$, 1 Hz, 37^0C) are only slightly less than those measured in control myocytes expressing GFP (68). Likewise, contractility and $[Ca^{2+}]_i$ transient amplitudes (1.8 mM $[Ca^{2+}]_o$, 1 Hz, 37^0C) are similar between WT and PLM-null myocytes (78). Only when the thermodynamic driving force for NCX1 is altered by varying $[Ca^{2+}]_o$ (0.6 or 5.0 mM) are the effects of PLM on myocyte contractility and $[Ca^{2+}]_i$ transients evident (45, 68, 69, 78, 90). The effects of PLM on Na^+-K^+-ATPase are also not apparent in myocytes under resting conditions: basal $[Na^+]_i$ is similar between wild-type and PLM-null myocytes (17, 80). Therefore, under resting conditions, PLM is functionally quiescent.

In the intact heart, β-adrenergic stimulation increases chronotropy leading to more frequent depolarizations and increased Na^+ entry. In addition, I_{Ca} and SERCA2 activity are also increased in response to β-adrenergic stimulation, resulting in increased Ca^{2+} entry and SR Ca^{2+} loading. Elevated SR Ca^{2+} content available for release largely accounts for the enhanced inotropy associated with β-adrenergic stimulation. Increased Ca^{2+} entry must be balanced by greater Ca^{2+} efflux mediated by forward NCX1, thereby bringing more Na^+ into the myocyte. This, if unchecked, will lead to cellular Na^+ and Ca^{2+} overload. Don Bers (18) hypothesized that β-adrenergic agonists increase PLM phosphorylation at serine^{68}, thereby activating Na^+-K^+-ATPase and resulting in lower $[Na^+]_i$. The lower $[Na^+]_i$ promotes Ca^{2+} efflux via NCX1, resulting in lower $[Ca^{2+}]_i$ transient and contraction amplitudes (18, 80). Indeed, when hearts in vivo are stressed with maximal doses of isoproterenol, inotropy (+dP/dt) rises to a peak within 2 minutes followed by decline in WT but not PLM-null hearts (Fig. 4). Therefore, when hearts are under duress, one of the major functions of PLM is to limit Na^+ and Ca^{2+} overload, thereby minimizing the risks of arrhythmogenesis apparently at the expense of reduced inotropy.

On the other hand, reduced cardiac contractility under conditions of fight or flight is clearly not in the best interests of the organism. In 2006 we proposed a coordinated paradigm in which PLM, upon phosphorylation at serine^{68}, enhances Na^+-K^+-ATPase activity to minimize risks of arrhythmogenesis but inhibits Na^+/Ca^{2+} exchanger to preserve inotropy during stress (87). Our recent experiments provide support for this hypothesis. In PLM-null hearts in which isoproterenol has little-to-no effects on Na^+-K^+-ATPase (17, 80), injection of recombinant adeno-associated virus (serotype 9) expressing the phosphomimetic PLM S68E mutant (rAAV9-S68E) directly into the LV resulted in expression of the mutant protein after 4-5 weeks. PLM S68E mutant inhibits NCX1 but not Na^+-K^+-ATPase (70). Isoproterenol stimulation resulted in similar

increases in $[Na^+]_i$ but higher +dP/dt in PLM-null hearts expressing PLM S68E mutant when compared to PLM-null hearts expressing GFP (Fig. 5). Therefore, inhibition of NCX1 by phosphorylated PLM preserves cardiac contractility under stressful situations.

FXYD Proteins in Aging, Exercise and Disease

PLM expression is 2-fold higher in neonatal rabbit ventricular membranes and declines within 10 days to the level observed in adults (71). The decrease in PLM expression with postnatal maturation is concurrent with reduction in Na^+-K^+-ATPase and Na^+/Ca^{2+} exchanger (71) and suggests tight coordination of PLM with the 2 ion transporters. With aging, expression of PLM in sedentary rat skeletal muscle is not altered but the level of α1-subunit of Na^+-K^+-ATPase that co-immunoprecipitates with PLM increases 3-fold (61). There are no detectable changes of association of α2-subunit of Na^+-K^+-ATPase with PLM with aging (61).

Acute exercise (treadmill running) in rats increases sarcolemmal PLM in skeletal muscle by 200-350% due to translocation, but phosphorylation at serine[68] appears not the be altered (60). When senescent rats (26 months-old) are subjected to endurance treadmill running for 13-14 weeks, PLM in skeletal muscle is increased by 150% when compared to sedentary senescent rats (61). In addition, increased association of PLM with α1-subunit of Na^+-K^+-ATPase in skeletal muscle of senescent rats (as compared to young rats) is decreased with endurance treadmill running (61).

In 2002, using cDNA microarrays containing 86 known genes and 989 unknown cDNAs, Sehl et al. (64) are the first to report that PLM is 1 of only 19 genes to increase after myocardial infarction (MI) in the rat. We confirmed that PLM protein levels increased 2.4- and 4-fold at 3 and 7 days post-MI, respectively, in the rat (89). PLM overexpression may very well explain the depression in both Na^+-K^+-ATPase (20) and Na^+/Ca^{2+} exchanger (19, 86) activities observed in

the post-MI rat model. In rat hearts subjected to acute ischemia, PLM is phosphorylated which leads to profound activation of sarcolemmal Na^+-K^+-ATPase (24). In isolated perfused mouse hearts subjected to ischemia/reperfusion, protection against infarction by sildenafil is associated with increased PLM phosphorylation at serine^{69} which enhances Na^+-K^+-ATPase activity during reperfusion (41). Increased Na^+-K^+-ATPase activity during acute ischemia ± reperfusion is critical in maintaining $[Na^+]_i$ homeostasis in order to minimize the adverse effects of elevated $[Na^+]_i$ on contractility and arrhythmogenesis. In human heart failure, protein levels of PLM in LV homogenates are reduced by 24% (8). In a rabbit model of volume overload heart failure which is prone to arrhythmias (57), expression of PLM is reduced by 42-48% but phosphorylation at serine^{68} is dramatically increased (8). Thus, both altered expression and phosphorylation of PLM have been observed in various cardiac disease models. In this context, it is very relevant to note that the 2 classes of drugs that have been clinically proven to be efficacious in human heart failure, β-adrenergic blockers (lowering PKA activity) and angiotensin converting enzyme inhibitors (reducing PKC activity), both have PLM as a common target.

Sepsis is a major clinical problem which is characterized by profound hypotension, systemic vasodilatation and depression in cardiac contractility. A wide range of inflammatory cascades is activated during systemic sepsis (54). Increased nitric oxide (NO) by inducible NO synthase (iNOS) has been suggested to cause depressed cardiac contractility in sepsis (26). In this light, Helge Rasmussen demonstrated that NO stimulates Na^+-K^+-ATPase (22, 83) and Mike Shattock reported (in abstract form) that this is dependent on PLM. Acceleration of Na^+-K^+-ATPase activity by PLM may account for hyperpolarization and relaxation of vascular smooth muscle (vasodilatation) in addition to depression of cardiac contractility.

PLM has also been implicated in other diseases. For example, PLM is downregulated in layer II/III stellate neurons in patients with schizophrenia (30). Rett syndrome, an X-linked neuro-development disorder that ranks as the second most prevalent cause of mental retardation in girls (49), is due to heterozygous de novo mutations in the methyl-CpG-binding protein 2 (MeCP2) gene. MeCP2 normally represses PLM transcription through direct interactions with sequences in the PLM promoter. In patients with Rett syndrome and MeCP2-null mice, PLM is elevated in neurons in the frontal cortex and cerebellum (16, 33). Increasing neuronal PLM expression is sufficient to reduce dendritic arborization and spine formation, hallmarks of neuropathology in patients with Rett syndrome.

Dominant-negative mutation in FXYD2 (γ-subunit of Na^+-K^+-ATPase) causes defective routing to the plasma membrane and is the cause of primary renal hypomagnesemia (44). Increased MAT-8 (FXYD3) expression is associated with tumor progression in human breast, prostate and colorectal cancers (84). Likewise, dysadherin (FXYD5) expression is altered in a wide variety of human cancers, including but not limited to breast, gastrointestinal stromal, head and neck, papillary thyroid, colorectal, non-small cell lung, and testicular cancers, and also epitheliod sarcoma and malignant melanoma (50).

Future Directions

The physiological role of PLM on regulation of L-type Ca^{2+} channel needs to be established in its natural environment. The stoichiometry of interaction between PLM and Na^+-K^+-ATPase, PLM and Na^+/Ca^{2+} exchanger, and PLM and L-type Ca^{2+} channel, remains to be determined in cardiac myocytes. The role of PLM in regulating cardiac contractility in vivo, both in health and disease states, needs further investigation. This will likely require development of novel genetic models. The effects of oxidative stress and NO on both PLM and

Na^+-K^+-ATPase are just beginning to be addressed. For effective but specific drug targeting, the precise molecular interactions between PLM and Na^+-K^+-ATPase, and PLM and Na^+/Ca^{2+} exchanger need to be mapped out.

Conclusion

FXYD proteins are emerging not only as novel endogenous regulators of ion transport but also as important targets in many human diseases including neurological, cardiac and renal diseases and a wide variety of cancers. Phospholemman (FXYD1) regulates Na^+-K^+-ATPase, Na^+/Ca^{2+} exchanger and possibly L-type Ca^{2+} channel in the heart. Its effects on in vivo cardiac contractility are complex and remain to be clarified. When hearts are subjected to stress, phospholemman minimizes risks of arrhythmogenesis and preserves inotropy. Elucidating the mechanisms by which alterations or mutations of FXYD proteins are involved in human diseases will undoubtedly provide novel and rational therapeutic targets.

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Figure Legends.

Figure 1. Molecular model of phospholemman. Nuclear magnetic resonance studies of highly purified phospholemman in micelles revealed 4 helices of the protein with a single transmembrane domain (after Francesca Marassi)(23, 76). The FXYD motif is in the extracellular domain and the important serine^{63} and serine^{68} are in the cytoplasm.

Figure 2. Phospholemman co-localizes with α1-subunit of Na^+-K^+-ATPase. Indirect immunofluorescence of adult rat ventricular myocytes doubly labeled with mouse monoclonal antibody against α1-subunit of Na^+-K^+-ATPase (A) and rabbit polyclonal anti-PLM antibody (B) are shown. Primary antibodies are visualized with Alexa Fluor 488-labeled goat anti-mouse IgG (A) and Alexa Fluor 594- labeled goat anti-rabbit IgG (B). Note the orange color in the merged image (C), indicating co-localization of PLM and Na^+-K^+-ATPase. Bar = 5 μm.

Figure 3. Cardiac excitation-contraction coupling. Membrane depolarization is initiated by opening of the Na^+ channel (not shown) with Na^+ entry. Extracellular Ca^{2+} enters via L-type Ca^{2+} channel (I_{Ca}) and Na^+/Ca^{2+} exchanger (NCX1), causing Ca^{2+} release from the ryanodine receptor (RyR) in the sarcoplasmic reticulum (SR). Ca^{2+} binds to troponin and initiates myofilament contraction. During diastole, Ca^{2+} is pumped back to the SR by SR Ca^{2+}-ATPase (SERCA) under the control of phospholamban (PLB). A small amount of Ca^{2+} is also taken up by the mitochondrial Ca^{2+} uniporter. The amount of Ca^{2+} that has entered during systole is extruded by Na^+/Ca^{2+} exchanger and to a lesser extent, by sarcolemmal Ca^{2+}-ATPase. Na^+ that has entered via Na^+ channel and Na^+/Ca^{2+} exchanger is pumped out by Na^+-K^+-ATPase. Repolarization is mediated by opening of K^+ channels (only the transient outward K^+ current I_{to} responsible for early repolarization is shown). Phospholemman (PLM) associates with and is an endogenous regulator of Na^+/Ca^{2+} exchanger, Na^+-K^+-ATPase and possibly L-type Ca^{2+} channel. Na^+/Ca^{2+} exchanger is depicted as operating in the forward mode (Ca^{2+} efflux) in the sarcolemma and reverse mode (Ca^{2+} influx) in the t-tubules. Broken arrows point to ion transporters, ion channels and myofilaments that are altered after myocardial infarction.

Figure 4. Effects of activation of Na^+-K^+-ATPase by phosphorylated phospholemman on β-adrenergic response in vivo. Shown are normalized in vivo hemodynamics (+dP/dt) of anesthetized wild-type (•; n=9) and phospholemman-null (□; n=14) mice after stimulation with 25 ng of

isoproterenol. Note time-dependent decline of +dP/dt in wild-type but not phospholemman-null hearts. Phospholemman phosphorylated at serine^{68} activates Na^+-K^+-ATPase (17, 80), leading to decreases in $[Na^+]_i$ in wild-type but not phospholemman-null cardiac myocytes (18, 80).

Figure 5. Effects of inhibition of Na^+/Ca^{2+} exchanger by phosphorylated phospholemman on β-adrenergic response in vivo. Left ventricles of phospholemman-null (KO) mice are injected with recombinant adeno-associated virus, serotype 9, expressing either green fluorescent protein (GFP)(□; n=5) or the phosphomimetic phospholemman S68E mutant (•; n=7). S68E mutant inhibits Na^+/Ca^{2+} exchanger but has no effect on Na^+-K^+-ATPase (69, 70, 87). Five weeks after virus injection, in vivo hemodynamics (+dP/dt) are measured in anesthetized mice (80). Note with increasing doses of isoproterenol, KO-S68E hearts contract significantly better than KO-GFP hearts. Since isoproterenol has no effect on Na^+-K^+-ATPase in phospholemman-null myocytes (17, 80), $[Na^+]_i$ is similar between KO-S68E and KO-GFP hearts (data not shown). Enhanced contractility in KO-S68E hearts is therefore due to inhibition of Na^+/Ca^{2+} exchanger by the S68E mutant.