Coordinated Regulation of Cardiac Na^+/Ca^{2+} Exchanger and Na^+-K^+-ATPase by Phospholemman (FXYD1)

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Abstract

Phospholemman (PLM) is the founding member of the FXYD family of regulators of ion transport. PLM is a 72-amino acid protein consisting of the signature PFXYD motif in the extracellular N terminus, a single transmembrane (TM) domain, and a C-terminal cytoplasmic tail containing three phosphorylation sites. In the heart, PLM co-localizes and co-immunoprecipitates with Na^+-K^+-ATPase, Na^+/Ca^{2+} exchanger, and L-type Ca^{2+} channel. The TM domain of PLM interacts with TM9 of the α-subunit of Na^+-K^+-ATPase, while its cytoplasmic tail interacts with two small regions (spanning residues 248–252 and 300–304) of the proximal intracellular loop of Na^+/Ca^{2+} exchanger. Under stress, catecholamine stimulation phosphorylates PLM at serine68, resulting in relief of inhibition of Na^+-K^+-ATPase by decreasing Km for Na^+ and increasing Vmax, and simultaneous inhibition of Na^+/Ca^{2+} exchanger. Enhanced Na^+-K^+-ATPase activity lowers intracellular Na^+, thereby minimizing Ca^{2+} overload and risks of arrhythmias. Inhibition of Na^+/Ca^{2+} exchanger reduces Ca^{2+} efflux, thereby preserving contractility. Thus, the coordinated actions of PLM during stress serve to minimize arrhythmogenesis and maintain inotropy. In acute cardiac ischemia and chronic heart failure, either expression or phosphorylation of PLM or both are altered. PLM regulates important ion transporters in the heart and offers a tempting target for development of drugs to treat heart failure.

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15.1 Introduction

The cardiac Na^+/Ca^{2+} exchanger (NCX1) is an important sarcolemmal ion transporter in the heart (Bers 2002). During most of the cardiac excitation-contraction (EC) cycle, NCX1 operates in the forward or Ca^{2+} efflux (3 Na^+ in: 1 Ca^{2+} out) mode. During systole, when the membrane potential (E_m) exceeds the equilibrium potential of NCX1 (E_{NaCa}), Ca^{2+} influx (3 Na^+ out: 1 Ca^{2+} in) is favored. This unique capability of NCX1 to modulate both Ca^{2+} influx and efflux during EC confers upon it the ability to regulate both contraction and relaxation. Altered expression and activity of NCX1 has been postulated to account for contractile dysfunction in many cardiac disease models, including human cardiomyopathy (Sipido et al. 2002). In addition, elevated NCX1 activity has been proposed as a mechanism for arrhythmogenesis in heart failure (Pogwizd et al. 2001). NCX1 therefore is a tempting target for drug development in therapy of heart failure, arrhythmias, and cardiomyopathy (Sipido et al. 2002; Hasenfuss and Schillinger 2004).

NCX1 is a 938-amino acid (939 amino acid in the rat) protein consisting of an extracellular N-terminal domain comprising the first five transmembrane (TM) segments, a large intracellular loop (residues 218–764), and an intracellular C-terminal domain comprising the last four TM segments (Fig. 15.1) (Nicoll et al. 1999; Philipson and Nicoll 2000). The α-repeats in TM segments 2, 3, and 7 of NCX1 are important in ion transport activity (Nicoll et al. 1996; Iwamoto et al. 2000), while the large intracellular loop contains the regulatory domains of the exchanger (Li et al. 1991; Levitsky et al. 1994; Maack et al. 2005). Specifically, the exchange inhibitory peptide (XIP) region (residues 219–238) (Li et al. 1991), the proximal linker domain (residues 259–370), the Ca^{2+}-binding domains (CBD) 1 (residues 371–508) (Levitsky et al. 1994) and 2 (residues 501–689) (Hilge et al. 2006), and the interaction site for endogenous XIP (residues 562–679) (Maack et al. 2005) all reside in the intracellular loop. Although the structures of CBD1 and CBD2 have been elucidated (Hilge et al. 2006, 2009; Nicoll et al. 2006; Ottolia et al. 2009), the three-dimensional structures of proximal linker domain and the distal intracellular loop remain unsolved.

Although NCX1 is a key Ca^{2+} transporter in the heart, remarkably little is known about its functional regulation (Blaustein and Lederer 1999; Philipson and Nicoll 2000; DiPolo and Beauge 2006). In 2002, we first speculated that phospholemman (PLM), the founding member of the FXYD family of small regulators of ion transport (Sweadner and Rael 2000), modulates NCX1 activity in the heart (Song et al. 2002). In 2003, we demonstrated that overexpression of PLM in adult rat cardiac myocytes inhibits Na^+/Ca^{2+} exchange current (I_{NaCa}) (Zhang et al. 2003). In 2005, we confirmed in transfected HEK293 cells that PLM is indeed the first reported endogenous regulator of NCX1 (Ahlers et al. 2005). In 2006, we showed that despite similar NCX1 protein levels (Tucker et al. 2006), I_{NaCa} is higher in PLM-knockout (KO) myocytes (Zhang et al. 2006a). Our results provide unambiguous proof that PLM is the first endogenous regulator of NCX1. This chapter reviews the discovery, structure, and known functions of PLM; the molecular sites of interaction between PLM and NCX1 and between PLM and Na^+-K^+-ATPase; and finally the role of PLM in maintenance of cardiac contractility and decrease in risks of arrhythmogenesis under stressful conditions.

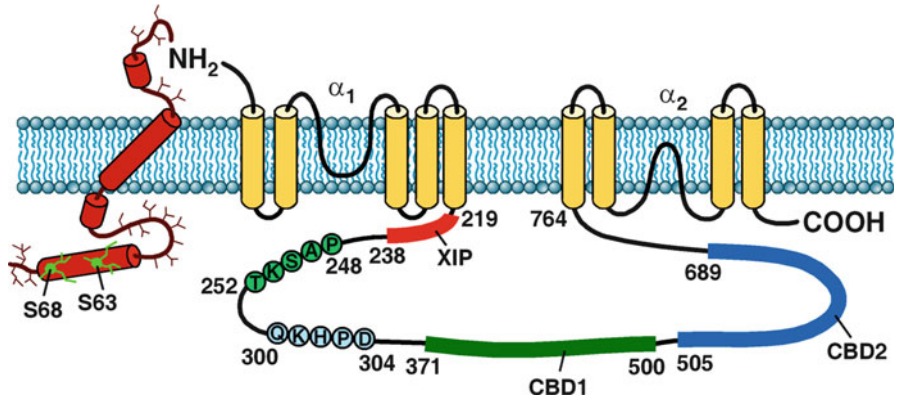

Fig. 15.1 *Molecular models of phospholemman and Na^+/Ca^{2+} exchanger. Left*: nuclear magnetic resonance studies of highly purified phospholemman in micelles reveal four helices of the protein with a single transmembrane (TM) domain. The FXYD motif is in the extracellular domain, and the physiologically important phosphorylation sites serine[63] and serine[68] are in the cytoplasm. *Right*: the mature Na^+/Ca^{2+} exchanger is modeled to consist of nine TM segments with two re-entrant loops (between TM2 and TM3 and between TM7 and TM8) as part of the conserved α-repeat motifs that are important in ion transport activity. The N terminus is extracellular and the C terminus is intracellular. Between TM5 and TM6 is a large intracellular loop (residues 218–764) which contains the regulatory domains of the exchanger. Specifically, the proximal linker domain (residues 218–358) which interacts with phospholemman, the exchange inhibitory peptide (XIP) region (residues 219–238), the two calcium-binding domains 1 (residues 371–500) and 2 (residues 505–689) connected in tandem by a short linker (residues 501–504), and the interaction site for endogenous XIP (residues 562–679) all reside within the intracellular loop. The two specific segments [residues 248–252 (PASKT) and residues 300–304 (QKHPD)] in the proximal linker domain responsible for inhibition of Na^+/Ca^{2+} exchanger by phospholemman are shown

15.2 Phospholemman: History

PLM was identified in 1985 as a 15-kDa sarcolemmal (SL) protein that is phosphorylated in response to isoproterenol and is distinct from the sarcoplasmic reticulum (SR) protein phospholamban (PLB) (Presti et al. 1985a). PLM is also phosphorylated by α-adrenergic agonists (Lindemann 1986) and protein kinase (PK) C (Presti et al. 1985b). The complete protein sequence of PLM has been determined and the cDNA cloned (Palmer et al. 1991). In humans, PLM gene is localized to chromosome 19q13.1 (Chen et al. 1997).

Early studies based on PLM overexpression in Xenopus oocytes suggest that PLM is a hyperpolarization-activated anion-selective channel (Moorman et al. 1995). In lipid bilayers, PLM forms a channel that is highly selective for taurine (Chen et al. 1998). In noncardiac tissues, PLM may function as a regulator of cell volume (Morales-Mulia et al. 2000; Davis et al. 2004). The function of PLM in the heart remained unknown until this century.

15.3 Phospholemman: Molecular Structure and Phosphorylation

PLM is synthesized as a 92-amino acid peptide with a 20-amino acid signal peptide at the N terminus. The mature PLM consists of 72 amino acids with a calculated molecular weight of 8,409 although the protein migrates at 15 kDa on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. The extracellular N terminus contains 17 amino acids which includes the PFXYD motif; a single transmembrane (TM) domain comprises of 20 amino acids, while the remaining 35 amino acids form the cytoplasmic tail. The C-terminal cytoplasmic tail of dog, human, rabbit, and rat PLM contains three serines (at residues 62, 63, and 68) and one threonine (at residue 69), but threonine[69] is replaced by serine in mouse PLM (Sweadner and Rael 2000). When reconstituted in liposomes, the TM domain of PLM is an α-helix with a maximum tilt of 15–17° (Beevers and Kukol 2006; Franzin et al. 2007). Highly purified PLM in model micelles

consists of four α-helices: H1 (residues 12–17) is in the extracellular domain, H2 (residues 22–38) is the main TM helix followed by a short H3 (residues 39–45), and H4 (residues 60–68) in the C terminus is connected to H3 by a flexible linker (Fig. 15.1) (Teriete et al. 2007). Synthetic peptide encompassing the TM domain of PLM forms tetramers when reconstituted in liposomes (Beevers and Kukol 2006). In transfected human embryonic kidney (HEK) 293 cells overexpressing PLM, fluorescence resonance energy transfer (FRET) studies suggest PLM forms oligomers consisting of 3–4 PLM molecules (Bossuyt et al. 2006, 2009; Song et al. 2011).

In isolated rat diaphragm, PLM is phosphorylated at serine^{68} by PKA and at both serine^{63} and serine^{68} by PKC (Waalas et al. 1994). In vitro studies using synthetic PLM fragments suggest that serine^{63} and threonine^{69} may be additional phosphorylation targets for PKA and PKC, respectively (Fuller et al. 2009). By assessing the effects of serine mutants of PLM on I_{NaCa}, ~46% of serine^{68} and ~16% of serine^{63} in adult rat cardiac myocytes are estimated to be phosphorylated in the resting state (Song et al. 2005). When evaluated with phospho-specific anti-PLM antibodies (Rembold et al. 2005; Fuller et al. 2009), ~30–40% of PLM in adult rat cardiac myocytes (Zhang et al. 2006b; Fuller et al. 2009) and ~25% of PLM in guinea pig myocytes (Silverman et al. 2005) are phosphorylated under resting conditions. In transfected HEK293 cells overexpressing PLM, ~30–45% of PLM is phosphorylated in the basal state (Zhang et al. 2009).

15.4 Phospholemman and the FXYD Family of Regulators of Ion Transport

Members in the FXYD family are small, single membrane-spanning proteins involved in regulation of ion transport (Sweadner and Rael 2000). PLM (FXYD1) is the first cloned member. All FXYD family members contain the signature PFXYD motif in the N terminus. With the exception of γ-subunit of Na^+-K^+-ATPase (FXYD2), all other FXYD proteins contain potential phosphorylation sites in the C terminus. As a family, FXYD proteins are expressed in tissues involved in fluid and solute transport (kidney, colon, pancreas, mammary gland, liver, lung, prostate, and placenta) or are electrically excitable (cardiac and skeletal muscle, neural tissues). To date, there are at least 12 known FXYD proteins including mammary-associated tumor 8 kDa (MAT-8 or FXYD3), channel-inducing factor (CHIF or FXYD4), dysadherin (FXYD5; also known as RIC [related to ion channel]), phosphohippolin (FXYD6), FXYD7, and phospholemman shark (PLM-S or FXYD10). Notably, FXYD3 and FXYD5 are overexpressed in a variety of tumors and may be associated with tumor progression (Nam et al. 2007; Yamamoto et al. 2009).

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

PLM co-immunoprecipitates (Crambert et al. 2002; Fuller et al. 2004; Bossuyt et al. 2005; Wang et al. 2010a) and co-localizes (Silverman et al. 2005; Cheung et al. 2010) with α-subunits of Na^+-K^+-ATPase in mammalian hearts. When co-expressed with α- and β-subunits of Na^+-K^+-ATPase in Xenopus oocytes, PLM increases K_m for Na^+ and K^+ without affecting V_{max} of the enzyme (Crambert et al. 2002). In cardiac myocytes and homogenates, PLM reduces its apparent affinities for intracellular Na^+ (Despa et al. 2005; Bossuyt et al. 2009) and extracellular K^+ (Han et al. 2009) as well as decreases V_{max} (Fuller et al. 2004; Zhang et al. 2006a; Bell et al. 2008; Wang et al. 2010a). Phosphorylation of PLM at serine^{68} results in increasing V_{max} (Fuller et al. 2004; Silverman et al. 2005; Wang et al. 2010a) while reducing the apparent K_m for Na^+ (Despa et al. 2005) but not for K^+ (Han et al. 2009) of cardiac Na^+-K^+-ATPase.

There are four isoforms of the catalytic α-subunits of Na^+-K^+-ATPase, and expression of each isoform is tissue and species dependent (Blanco and Mercer 1998). Whereas human (Zahler et al. 1993; McDonough et al. 1996) and rabbit (Bossuyt et al. 2005) hearts express α1- (ouabain-resistant),

α2-, and α3-isoforms, rodent hearts express only α1- and α2-isoforms of Na^+-K^+-ATPase. PLM co-immunoprecipitates all three α-subunits in human and rabbit (Bossuyt et al. 2005) and α1- and α2-subunits in mouse (Wang et al. 2010a) and bovine (Crambert et al. 2002), but only α1-subunit of Na^+-K^+-ATPase in rat (Fuller et al. 2004) and guinea pig (Silverman et al. 2005) hearts. It is important to note that co-immunoprecipitation of PLM with α-subunits of Na^+-K^+-ATPase does not require the presence of NCX1 in cardiac membranes (Wang et al. 2010a).

Exploiting the differential ouabain sensitivity between α1- and α2-subunits of Na^+-K^+-ATPase, currents due to Na^+-K^+-ATPase (I_{pump}) can be separated into that due to α1- ($I_{\alpha1}$) and α2-subunit ($I_{\alpha2}$) activities. $I_{\alpha1}$ represents ~ 82–88% and ~73% of I_{pump} in mouse (Berry et al. 2007; Wang et al. 2010a) and guinea pig cardiac myocytes (Silverman et al. 2005), respectively. By measuring I_{pump} in adult mouse and guinea pig cardiac myocytes at baseline and after isoproterenol stimulation, it appears that PLM regulates the activity of α1- but not α2-subunit of Na^+-K^+-ATPase. This conclusion is based on derivation of $I_{\alpha2}$ from the difference of two large numbers ($I_{pump} - I_{\alpha1}$), and the method may not have the requisite sensitivity to detect small changes in $I_{\alpha2}$ in response to isoproterenol stimulation. Using "SWAP" mouse (Dostanic et al. 2004) in which the ouabain affinities of the α-subunits are reversed, PLM regulates the apparent K_m for Na^+ of both α1- and α2-subunits of Na^+-K^+-ATPase (Bossuyt et al. 2009). It is therefore plausible but not definitively proven that in wild-type (WT) hearts, PLM regulates the activities of both α1- and α2-subunits of Na^+-K^+-ATPase.

15.6 Molecular Interactions Between Phospholemman and Na^+-K^+-ATPase

Early studies using mutational analysis suggest that FXYD proteins (FXYD2, 4 and 7) interact with TM9 segment of Na^+-K^+-ATPase (Li et al. 2004). Covalent cross-linking and co-immunoprecipitation studies demonstrate that the TM segment of PLM is close to TM2 of Na^+-K^+-ATPase (Lindzen et al. 2006). Based on crystal structure of Ca^{2+}-ATPase in the E_1-ATP bound conformation, the TM segment of PLM is modeled to dock in the groove between TM segments 2, 6, and 9 of the α-subunit of Na^+-K^+-ATPase. High-resolution (2.4 Å) crystal structure of shark rectal gland Na^+-K^+-ATPase in the E2.2 K^+. P_i state indicates that FXYD proteins interact almost exclusively with the outside of TM9 of the α-subunit (Shinoda et al. 2009). The FXYD motif stabilizes interactions between α- and β-subunits of the Na^+ pump.

Phosphorylation of PLM-S causes it to dissociate from shark Na^+-K^+-ATPase (Mahmmoud et al. 2000). By contrast, phosphorylated PLM remains associated with the α-subunit of Na^+-K^+-ATPase (Silverman et al. 2005; Bossuyt et al. 2009). Nuclear magnetic resonance studies of highly purified PLM reconstituted in micelles show no major conformational changes on phosphorylation of serine[68] (Teriete et al. 2009). By contrast, in transfected HEK293 cells co-expressing PLM and Na^+-K^+-ATPase, FRET demonstrates decrease in interaction between phosphorylated PLM and Na^+-K^+-ATPase (Bossuyt et al. 2006; 2009). The conclusion that PLM phosphorylation alters its conformation is given additional support by FRET which shows phosphomimetic mutants of PLM reduce the apparent affinity of interaction between Na^+-K^+-ATPase and PLM (Song et al. 2011). It is likely that phosphorylation of PLM results in subtle changes in its conformation and alters its physical interaction with Na^+-K^+-ATPase.

15.7 Functional Significance of Na^+-K^+-ATPase Regulation by PLM in Resting Heart

A major contribution to the elucidation of the physiological function of PLM in the heart is the generation of PLM-KO mice (Jia et al. 2005). Compared to WT hearts, congenic PLM-KO hearts express similar levels of α1-, α2-, β1-, and β2-subunits of Na^+-K^+-ATPase, NCX1, SR Ca^{2+}-ATPase (SERCA2), PLB, and calsequestrin

(Tucker et al. 2006; Bell et al. 2008). However, Na^+-K^+-ATPase enzymatic activity (Bell et al. 2008) and I_{pump} (Song et al. 2008; Wang et al. 2010a) are higher in PLM-KO hearts, as expected from relief of tonic inhibition by PLM. Higher Na^+-K^+-ATPase activity in PLM-KO hearts would be expected to lower intracellular Na^+ concentration ($[Na^+]_i$), thereby thermodynamically favoring Ca^{2+} efflux via NCX1 and resulting in lower cardiac contractility (Bell et al. 2008). However, basal $[Na^+]_i$ was similar between WT and PLM-KO cardiac myocytes (Despa et al. 2005; Wang et al. 2010a). In isolated cardiac myocytes paced to contract at 1 Hz and extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) of 1.8 mM, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) transient and myocyte contraction amplitudes are similar between WT and PLM-KO myocytes (Tucker et al. 2006). Finally, in vivo myocardial contractility assessed by echocardiography and cardiac catheterization shows no differences in ejection fraction, $^+dP/dt$ and $-dP/dt$ between WT and PLM-KO hearts (Bell et al. 2008; Wang et al. 2010a) in the resting state. These observations indicate that under resting conditions, the regulatory effects of PLM on Na^+-K^+-ATPase in the heart are not discernible and PLM is functionally quiescent.

The paradox that Na^+-K^+-ATPase activity is higher but cardiac contractility is similar between WT and PLM-KO hearts may be reconciled by the following considerations: First, there may be a distinct pool of Na^+-K^+-ATPase not regulated by PLM but intimately involved in regulation of cardiac contractility. For example, in adult mouse and rat cardiac myocytes, the α2-subunit of Na^+-K^+-ATPase is preferentially distributed in the t-tubules (Berry et al. 2007; Swift et al. 2007) and is involved with regulation of Ca^{2+} (James et al. 1999) and contractility (Swift et al. 2007). PLM appears not to regulate α2-subunit of Na^+-K^+-ATPase (Silverman et al. 2005; Wang et al. 2010a) although this point is controversial (Bossuyt et al. 2009). Second, maintenance of resting $[Na^+]_i$ under basal conditions may only require a fraction of Na^+-K^+-ATPase activity. Assuming the 1:1 stoichiometry of PLM:Na^+-K^+-ATPase observed in transfected HEK293 cells (Bossuyt et al. 2009), the 25–40% of PLM phosphorylated in resting cardiac myocytes (Silverman et al. 2005; Zhang et al. 2006b; Fuller et al. 2009) may unencumber enough Na^+-K^+-ATPase for basal $[Na^+]_i$ to be maintained at similar levels between WT and PLM-KO myocytes. Third, resting $[Na^+]_i$ in mouse myocytes of 6–12 mM (Despa et al. 2005; Wang et al. 2010a, 2011) is close to the K_m for Na^+ (~10 mM) of Na^+-K^+-ATPase (Despa et al. 2002; Zhang et al. 2006b). Since PLM regulates Na^+-K^+-ATPase by both K_m for Na^+ (Despa et al. 2005) and V_{max} (Zhang et al. 2006b; Bell et al. 2008) mechanisms, the effects of PLM on Na^+-K^+-ATPase activity at resting $[Na^+]_i$ may be rather small. This hypothesis is supported by the observation that I_{pump} is higher in PLM-KO myocytes under high but not low pipette $[Na^+]$ conditions (Wang et al. 2010a).

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

When dog PLM is overexpressed (1.4- to 3.5-fold) in adult rat cardiac myocytes by adenovirus-mediated gene transfer, expression of α1- and α2-subunits of Na^+-K^+-ATPase, NCX1, SERCA2, and calsequestrin is not affected (Song et al. 2002; Zhang et al. 2003, 2006a). As expected, I_{pump} is decreased in rat myocytes overexpressing PLM, primarily due to reduction in V_{max} rather than changes in apparent K_m for Na^+ or K^+ (Zhang et al. 2006b). Inhibition of Na^+-K^+-ATPase activity would be expected to enhance myocyte contractility, similar to the mechanism of action of digitalis glycosides (Grupp et al. 1985). A totally unexpected but critical observation is that both contraction and $[Ca^{2+}]_i$ transient amplitudes (1 Hz, 5 mM $[Ca^{2+}]_o$) are lower, rather than higher, in myocytes overexpressing PLM (Song et al. 2002). By manipulating $[Ca^{2+}]_o$ and thus the thermodynamic driving force favoring Ca^{2+} influx or efflux via NCX1, the contraction phenotype of myocytes overexpressing PLM is reminiscent of that of myocytes in which NCX1 is downregulated (Tadros et al. 2002), leading us to speculate that PLM may directly inhibit NCX1 (Song et al. 2002). Follow-up studies demonstrate that PLM co-localizes with NCX1 in the sarcolemma and t-tubules (Zhang et al. 2003);

that PLM co-immunoprecipitates with NCX1 in rat, pig, and guinea pig cardiac membranes (Mirza et al. 2004; Ahlers et al. 2005; Wang et al. 2010b); that PLM overexpression inhibits I_{NaCa} (Zhang et al. 2003; Song et al. 2005) and PLM downregulation increases I_{NaCa} in rat cardiac myocytes (Mirza et al. 2004); and that I_{NaCa} is higher in PLM-KO compared to WT mouse myocytes despite no differences in NCX1 protein levels (Zhang et al. 2006a). The conclusion that PLM directly inhibits NCX1 is further supported by observations in transfected HEK293 cells co-expressing PLM and NCX1, in which both I_{NaCa} and Na^+-dependent Ca^{2+} uptake are depressed by the presence of PLM (Ahlers et al. 2005).

15.9 PLM Regulation of NCX1 Is not Mediated by Ca^{2+}-Dependent Activation

Regulation of NCX1 by PLM does not depend on synergistic interactions between CBD1 and CBD2 (Hilge et al. 2009; Ottolia et al. 2009; Giladi et al. 2010; John et al. 2011), thereby modulating Ca^{2+}-dependent activation of NCX1 (Matsuoka et al. 1995). First, I_{NaCa} measured in HEK293 cells expressing split NCX1 exchangers (comprising of N- or C-terminal domains with varying lengths of the intracellular loop) (Ottolia et al. 2001) in which both CBD1 and CBD2 are absent is still inhibited by PLM (Wang et al. 2006). Second, CBD1 (spanning residues 371–508) has no physical association with PLM (Wang et al. 2006). Finally, I_{NaCa} measured in HEK293 cells expressing NCX1-G503P mutant which lacks Ca^{2+}-dependent activation of NCX1 (Matsuoka et al. 1995) is still inhibited by PLM (Zhang et al. 2011).

15.10 Molecular Interactions Between PLM and Na^+/Ca^{2+} Exchanger

Glutathione S-transferase (GST) pull-down assay demonstrates that the intracellular loop but not the N or C terminus of NCX1 associates with PLM (Wang et al. 2006). Specifically, PLM binds to GST constructs fused to NCX1 loop segments encompassing residues 218–371 and 508–764 but not 371–508 (CBD1). Co-expressing split NCX1 exchangers (Ottolia et al. 2001) with PLM in HEK293 cells, PLM inhibits I_{NaCa} only when split exchangers contain residues 218–358 of the intracellular loop (Wang et al. 2006). PLM co-immunoprecipitates with N-terminal split exchanger only when it contains the intracellular loop segment spanning residues 218–358. These observations strongly indicate that PLM physically associates with the proximal linker domain (encompassing residues 218–358) of NCX1 and regulates I_{NaCa} via interacting with this region.

To further refine the region of NCX1 required for regulation by PLM, a family of overlapping NCX1 deletion mutants spanning the proximal linker domain is constructed and expressed in HEK293 cells. By mapping whether I_{NaCa} inhibition by PLM is preserved in overlapping NCX1 deletion mutants, it was deduced that two regions encompassing residues 238–270 and 300–328 must be present in order for PLM to regulate NCX1 (Zhang et al. 2009). Indeed, NCX1 mutants in which either residues 238–270 or residues 300–328 are deleted lose their ability to be regulated by PLM. Finally, GST constructs fused to residues 218–270 and 300–373 but not residues 250–300 physically associate with PLM. These observations indicate that two discrete regions (spanning residues 238–270 and 300–328) in the proximal linker domain of NCX1 interact with and are responsible for its regulation by PLM.

Alanine linker scanning was next used to pinpoint the residues in the proximal linker domain responsible for regulation of NCX1 by PLM. Mutating residues 248–252 (PASKT) or 300–304 (QKHPD) to alanine results in loss of I_{NaCa} inhibition by PLM in transfected HEK293 cells (Zhang et al. 2011). While mutating residue 301 alone completely abolishes PLM inhibition, single alanine mutation of residues 250–252, 300, or 302–304 results in partial reduction in inhibition. In addition, mutating residues 248–252 to alanine weakens the association with PLM, as demonstrated by GST pull-down assays. Taken together,

the results indicate that the cytoplasmic tail of PLM physically and functionally interacts with residues 248–252 (PASKT) and 300–304 (QKHPD) of NCX1 (Fig. 15.1).

Although the structure of proximal linker domain is unknown, one hypothetical model of the region encompassing residues 259–370 suggests that Val^{261} is in close proximity with Ala^{314} (Hilge et al. 2006). Consistent with this model and assuming one PLM molecule interacts with one NCX1 molecule, the two small regions spanning residues 248–252 and 300–324 required for I_{NaCa} inhibition by PLM are close to each other and suggest some "structure" of the proximal linker domain. Alternatively, if, as observed in model lipid membranes and heterologous expression systems, both NCX1 (Ren et al. 2008; John et al. 2011) and PLM (Beevers and Kukol 2007; Song et al. 2011) exist as dimers and oligomers, then one PLM molecule interacts with the region encompassing residues 248–252 of one NCX1 molecule while a second PLM molecule interacts with the region spanning residues 300–304 of the other NCX1 molecule. Whether NCX1 exists as dimers and oligomers in native cardiac membranes remains to be established.

15.11 Differences Between PLM Regulation of Na^+-K^+-ATPase and Na^+/Ca^{2+} Exchanger

There are major mechanistic differences between regulation of Na^+-K^+-ATPase and NCX1 by PLM. First, inhibition of Na^+-K^+-ATPase is relieved by PLM phosphorylation. By contrast, PLM phosphorylated at $serine^{68}$ inhibits NCX1 in transfected HEK293 cells (Zhang et al. 2006a). When expressed in WT rat (Song et al. 2005) or PLM-KO mouse myocytes (Song et al. 2008), the phosphomimetic PLM S68E mutant inhibits I_{NaCa} with no effect on I_{pump}. By contrast, the non-phosphorylable PLM S68A mutant inhibits I_{pump} but not I_{NaCa} in PLM-KO mouse myocytes. Second, the single TM segment of FXYD proteins interacts with TM segments of Na^+-K^+-ATPase. By contrast, association between PLM and NCX1 does not appear to involve the TM segments (Wang et al. 2006). Rather, regulation of NCX1 is mediated through the interaction between the cytoplasmic tail of PLM and intracellular loop of NCX1 (Fig. 15.1) (Song et al. 2005; Wang et al. 2006).

15.12 Regulation of Myocyte Contraction by PLM: Na^+-K^+-ATPase vs. NCX1

In PLM-KO myocytes expressing the phosphomimetic PLM S68E mutant, contraction and $[Ca^{2+}]_i$ transient amplitudes are decreased at 5.0 but not 1.8 mM $[Ca^{2+}]_o$ (Song et al. 2008). When the non-phosphorylable PLM S68A mutant is expressed, no changes in $[Ca^{2+}]_i$ transient and contraction amplitudes are observed at either $[Ca^{2+}]_o$. 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 of NCX1 rather than Na^+-K^+-ATPase.

On the other hand, when WT myocytes are Na^+ loaded by rapid pacing and isoproterenol exposure, relief of inhibition of Na^+-K^+-ATPase associated with PLM phosphorylation results in lowering of $[Na^+]_i$, $[Ca^{2+}]_i$ transient and contraction amplitudes (Fig. 15.2) (Despa et al. 2008; Wang et al. 2010a). In PLM-KO myocytes subjected to similar treatment, no enhancement of

before and after isoproterenol (1 μM) addition. There are eight WT (o) and six PLM-KO (•) myocytes. Note $\Delta[Na^+]_i$ (increase of $[Na^+]_i$ above baseline) reaches a peak after isoproterenol followed by a decline in WT myocytes. The time-dependent decline in $\Delta[Na^+]_i$ is due to relief of inhibition of Na^+-K^+-ATPase upon phosphorylation of phospholemman. The time-dependent decline in $[Na^+]_i$ in WT myocytes promotes increased Ca^{2+} efflux via Na^+/Ca^{2+} exchanger, leading to decreased $[Ca^{2+}]_i$ transient (**e**) and contraction (**d**) amplitudes. In PLM-KO myocytes, no increase Na^+-K^+-ATPase activity occurs following isoproterenol stimulation and $\Delta[Na^+]_i$ continues its monotonous rise, without any decrease in $[Ca^{2+}]_i$ transient and contraction amplitudes

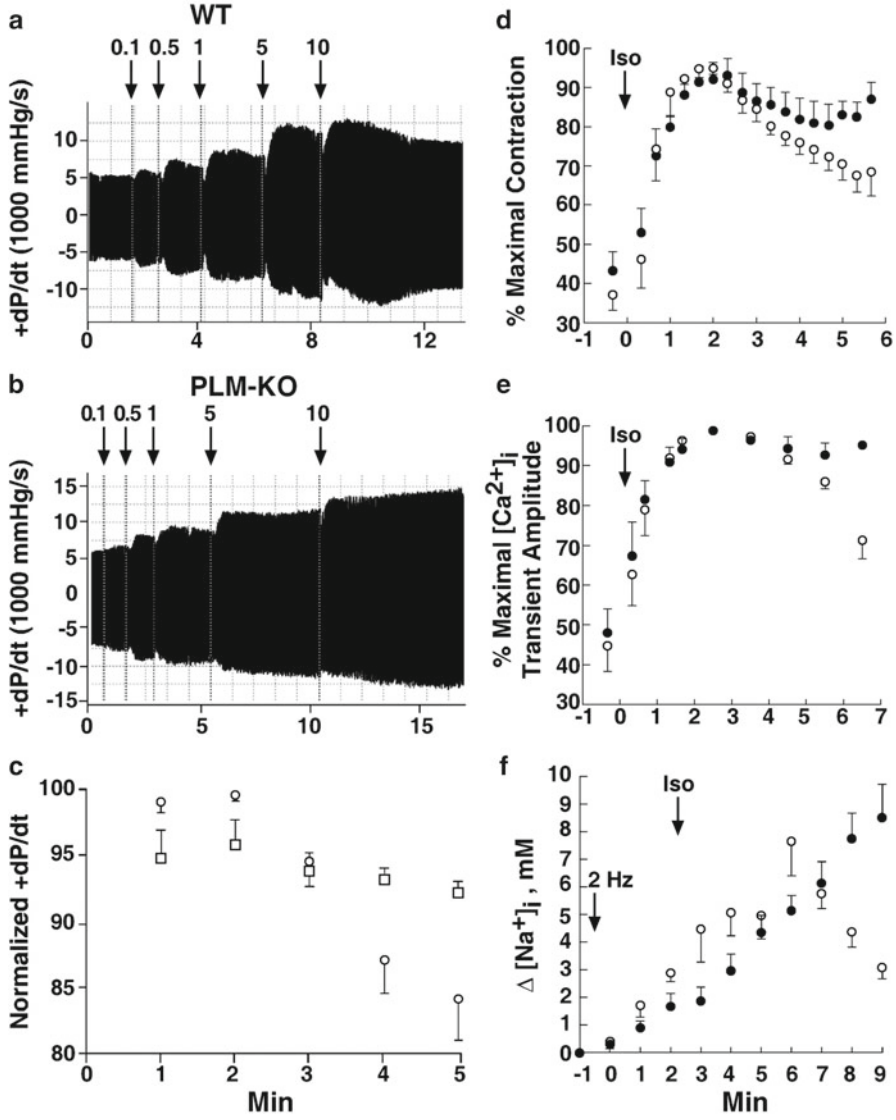

Fig. 15.2 *Disinhibiting Na^+-K^+-ATPase by phosphorylated phospholemman minimizes $[Ca^{2+}]_i$ and $[Na^+]_i$ overload and reduces risks of arrhythmogenesis in hearts under stress but at the apparent expense of reduced inotropy.* (**a**) First-time derivatives of left ventricular pressure rise ($^+dP/dt$) measured in an anesthetized, closed-chest wild-type (WT) mice are continuously measured, both at baseline and with increasing doses of isoproterenol (arrows; ng). Note $^+dP/dt$ increases to a maximum followed by time-dependent decline. (**b**) Absence of time-dependent decline in $^+dP/dt$ in phospholemman knockout (PLM-KO) after isoproterenol addition. (**c**) Time course of normalized $+dP/dt$ (means ± SE) following addition of maximal doses (10 or 25 ng) of isoproterenol to five WT (o) and eight PLM-KO (□) mice. (**d**) To elucidate the cellular mechanisms responsible for the time-dependent decline in $+dP/dt$ in WT hearts following isoproterenol stimulation, isolated myocytes are paced at 2 Hz, 37°C, and 1.8 mM $[Ca^{2+}]_o$. After reaching steady-state contraction amplitudes, isoproterenol (Iso; 1 μM) is added. There are six WT (o) and six PLM-KO (•) myocytes. Similar to intact hearts, in isolated myocytes stimulated with isoproterenol, contraction amplitude reaches a peak followed by rapid decline in WT but not PLM-KO myocytes. (**e**) $[Ca^{2+}]_i$ transient amplitudes are measured in paced, fura-2 loaded myocytes, before and after isoproterenol (1 μM) addition. There are ten WT (o) and six PLM-KO (•) myocytes. Note $[Ca^{2+}]_i$ transient amplitude reaches a peak after isoproterenol stimulation, followed by decline in WT but not PLM-KO myocytes. (**f**) $[Na^+]_i$ is measured in paced, sodium-binding benzofuran isophthalate (SBFI)-loaded myocytes,

Na^+-K^+-ATPase activity is observed, $[Na^+]_i$ continues to increase, and $[Ca^{2+}]_i$ transient and contraction amplitudes remain stable (Fig. 15.2). Thus, at the level of a single myocyte, PLM can be manipulated to regulate Na^+ and Ca^{2+} fluxes (and therefore $[Ca^{2+}]_i$ transients and contractility) by either Na^+/Ca^{2+} exchanger or Na^+-K^+-ATPase, depending on experimental conditions.

15.13 Functional Significance of Na^+/Ca^{2+} Exchanger Regulation by PLM at Rest

Inhibition of NCX1 activity by the ~30–40% phosphorylated PLM in resting hearts would be predicted to reduce Ca^{2+} efflux and secondarily increase SR Ca^{2+} load, resulting in enhanced contractility. However, in vivo cardiac function (Wang et al. 2010a) and in vitro myocyte contractility (Tucker et al. 2006) are similar between WT and PLM-KO mice, indicating that the regulatory effects of PLM on NCX1 are not detectable in hearts under basal conditions and PLM is functionally quiescent.

15.14 Phospholemman: A Novel Cardiac Stress Protein

Under stressful conditions when catecholamine levels are high, increased heart rate results in increased Na^+ entry. In addition, β-adrenergic agonists also enhance Ca^{2+} channel and SERCA2 activity, resulting in increased Ca^{2+} entry and SR Ca^{2+} loading. Increased Ca^{2+} entry must be balanced by greater Ca^{2+} efflux via NCX1, thereby bringing more Na^+ into the myocyte. Therefore, stress results in elevations in $[Na^+]_i$ and $[Ca^{2+}]_i$, with consequent increased risks of arrhythmogenesis. Phosphorylation of PLM by PKA relieves tonic inhibition of Na^+-K^+-ATPase, thereby lowering $[Na^+]_i$. The lower $[Na^+]_i$ promotes Ca^{2+} efflux via NCX1, thereby minimizing $[Ca^{2+}]_i$ overload. However, increased Ca^{2+} efflux results in decreased SR Ca^{2+} load, leading to reduced $[Ca^{2+}]_i$ transient and contraction amplitudes. Indeed, when stimulated with isoproterenol, $[Na^+]_i$, $[Ca^{2+}]_i$ transient and contraction amplitudes initially rise followed by rapid decline in WT but not PLM-KO myocytes (Fig. 15.2) (Despa et al. 2008; Wang et al. 2010a). Similarly, when hearts in vivo are stressed with maximal doses of isoproterenol, $^+dP/dt$ rises to a peak within 2 min followed by decline in WT but not PLM-KO hearts (Fig. 15.2). Thus, one of the major physiological functions of PLM (mediated by relief of inhibition of Na^+-K^+-ATPase) is to limit Na^+ and Ca^{2+} overload in hearts under duress, thereby minimizing risks of arrhythmogenesis but at the apparent expense of reduced contractility.

Decreased inotropy in fight or flight circumstances is clearly not in the best interests of the animal attempting to survive. What, if any, are the effects of PLM regulation of NCX1 in hearts under stress?

To eliminate the effects of Na^+-K^+-ATPase regulation by PLM, a model of "pure" NCX1 inhibition is engineered to evaluate the functional significance of NCX1 regulation by PLM in the heart. Recombinant adeno-associated virus, serotype 9 (rAAV9) expressing either the phosphomimetic PLM S68E mutant (rAAV9-S68E) or control green fluorescent protein (rAAV9-GFP) is injected into left ventricles (LV) of PLM-KO hearts (Fig. 15.3). After 5–6 weeks, ~40% of LV myocytes express the exogenous gene (Wang et al. 2011). Since S68E mutant inhibits I_{NaCa} but not I_{pump} (Song et al. 2008), and since PLM-KO myocytes do not exhibit regulation of Na^+-K^+-ATPase (Despa et al. 2005; Wang et al. 2010a), the effects of PLM regulation of NCX1 can be evaluated without the confounding influence of changes in Na^+-K^+-ATPase activity. Baseline cardiac output as evaluated by echocardiography is similar between PLM-KO hearts expressing S68E mutant or GFP (Wang et al. 2011), in agreement with previous conclusion that under resting conditions, PLM is functionally quiescent. When isoproterenol is added to simulate stressful conditions, PLM-KO hearts expressing S68E mutant have significantly higher contractility than those expressing GFP, despite <40% of LV expresses

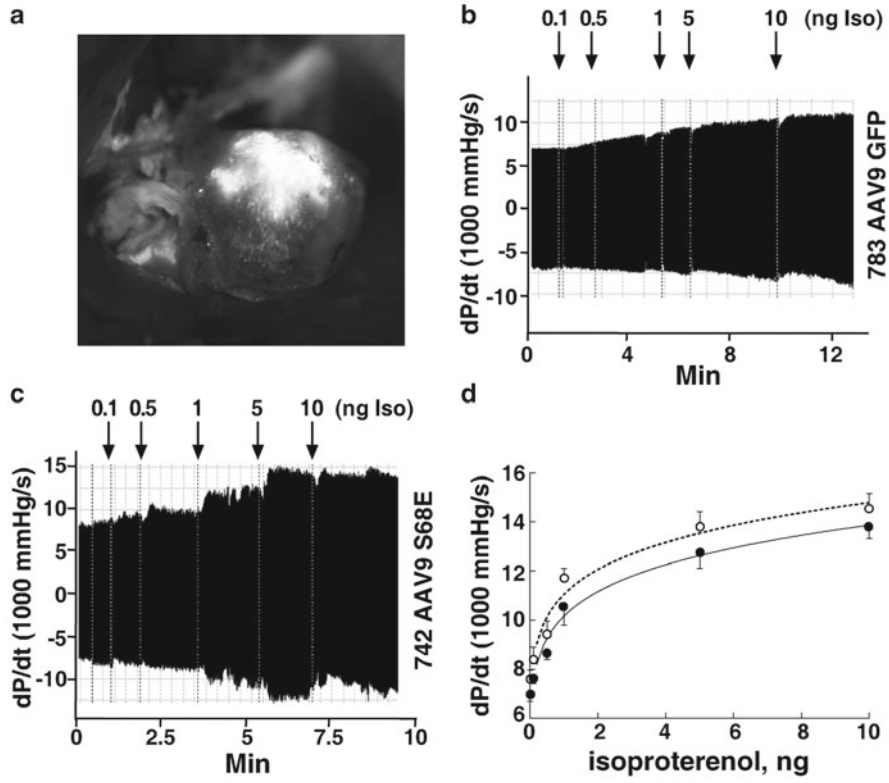

Fig. 15.3 *Inhibiting Na^+/Ca^{2+} exchanger by phosphorylated phospholemman preserves inotropy in hearts under stress.* (**a**) The apex, anterior, and posterior left ventricular (LV) wall of phospholemman knockout (PLM-KO) mice are injected with recombinant adeno-associated virus, serotype 9 (rAAV9), expressing either control green fluorescent protein (GFP) or GFP + phosphomimetic phospholemman S68E mutant (inhibits Na^+/Ca^{2+} exchanger but not Na^+-K^+-ATPase). The exogenous genes are expressed 5–6 weeks after injection as indicated by GFP fluorescence. About 40% of LV myocytes are successfully infected by rAAV9 injection. (**b**) First-time derivatives of LV pressure rise (+d*P*/d*t*) measured in an anesthetized, closed-chest PLM-KO mice previously injected with rAAV9-GFP, both at baseline and at increasing doses of isoproterenol (*arrows*, 0.1–10 ng). (**c**) +d*P*/d*t* in PLM-KO mice previously injected with rAAV9-S68E, both at baseline and at increasing doses of isoproterenol (arrows). Note there is no time-dependent decline in +d*P*/d*t* following isoproterenol addition, in contrast to the progressive decline in +d*P*/d*t* in WT hearts (Fig. 15.2a). This is consistent with lack of effect on Na^+-K^+-ATPase by S68E mutant. Note also +d*P*/d*t* is higher in PLM-KO heart expressing S68E mutant compared to that expressing control GFP (b). (**d**) Averaged maximal +d*P*/d*t* (means ± SE) achieved with each dose of isoproterenol in five PLM-KO hearts expressing S68E (o) and six PLM-KO hearts expressing GFP (•). Two-way ANOVA indicates significant ($P < 0.047$) differences in +d*P*/d*t* between the two groups

the mutant (Fig. 15.3). Consistent with NCX1 inhibition in PLM-KO myocytes expressing S68E, increase in $[Na^+]_i$ after isoproterenol stimulation is smaller (when compared to PLM-KO myocytes expressing GFP), due to decreased Na^+ entry via forward NCX1. Therefore, a second major function of PLM (mediated by inhibition of NCX1) is to maintain inotropy under stress.

15.15 Phospholemman in Cardiac Diseases

In the rat myocardial infarction (MI) model, PLM is one of 19 genes (in a cDNA microarray containing 86 known genes and 989 unknown cDNAs) to increase after MI (Sehl et al. 2000).

PLM protein levels increase 2.4- and 4-fold at 3 and 7 days post-MI, respectively, in the rat (Zhang et al. 2006b). PLM overexpression may explain decreased NCX1 (Dixon et al. 1992a; Zhang et al. 1996) and Na^+-K^+-ATPase (Dixon et al. 1992b) activities in the post-MI rat model. In rat hearts subjected to acute ischemia, PLM is phosphorylated with resultant increase in Na^+-K^+-ATPase activity (Fuller et al. 2004). In mouse hearts subjected to ischemia/reperfusion, protection against infarction by sildenafil is associated with increased PLM phosphorylation at serine[69] that enhances Na^+-K^+-ATPase activity during reperfusion (Madhani et al. 2010). Maintaining $[Na^+]_i$ homeostasis during ischemia and reperfusion by enhanced Na^+-K^+-ATPase activity minimizes the deleterious effects of elevated $[Na^+]_i$ on contractility and arrhythmogenesis. In a rabbit model of volume overload heart failure that is prone to arrhythmias, PLM expression is reduced by 42–48%, but serine[68] phosphorylation is dramatically increased (Bossuyt et al. 2005). In human heart failure, PLM in left ventricles is reduced by 24% (Bossuyt et al. 2005). Thus, both expression and phosphorylation state of PLM are altered in various cardiac disease models, making PLM a rational therapeutic target. In this context, it is very relevant to note that the two classes of drugs that are clinically efficacious in the treatment of human heart failure, β-adrenergic blockers (targeting PKA) and angiotensin-converting-enzyme inhibitors (targeting PKC), both have PLM as a common target.

15.16 Future Directions

Regulation of L-type Ca^{2+} channel by PLM has been demonstrated in transfected HEK293 cells (Wang et al. 2010b). The possibility that PLM regulates L-type Ca^{2+} channel in the heart needs to be unequivocally proved since this adds tremendous complexity to the design and interpretation of experiments. The stoichiometry of interaction between PLM and Na^+-K^+-ATPase, PLM and NCX1, and PLM and L-type Ca^{2+} channel in cardiac tissues needs to be determined. The structure of the proximal linker domain of NCX1 crucial for its regulation by PLM needs to be elucidated. Whether PLM exists as monomers or oligomers in cardiac membranes requires clarification. The effects of oxidative stress (Figtree et al. 2009), NO (William et al. 2005), and glutathionylation (Bibert et al. 2011) on Na^+-K^+-ATPase and FXYD proteins are just beginning to emerge. Methods needed to be developed to measure $[Na^+]_i$ in vivo to prove that the time-dependent decrease in contractility in WT hearts stimulated with isoproterenol is associated with decreased $[Na^+]_i$ due to enhanced Na^+-K^+-ATPase activity. Finally, the role of PLM in regulating in vivo contractility, both in health and disease, needs to be further delineated and will likely require novel genetic models.

15.17 Conclusion

Phospholemman, the inaugural member of FXYD family, regulates Na^+-K^+-ATPase, Na^+/Ca^{2+} exchanger, and possibly L-type Ca^{2+} channel in the heart. In the resting state, phospholemman is functionally quiescent in that its regulatory effects on Na^+-K^+-ATPase and Na^+/Ca^{2+} exchanger on cardiac contractility are not manifested. Under stress when catecholamine levels are high, the coordinated actions of phospholemman on Na^+-K^+-ATPase and Na^+/Ca^{2+} exchanger minimize the risks of arrhythmogenesis and preserve inotropy, respectively. Phospholemman expression and phosphorylation are altered in ischemic heart disease and heart failure. Phospholemman is likely a useful target for drug therapy.

15.18 Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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