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The Human Phospholamban Arg14-Deletion Mutant Localizes to Plasma Membrane and Interacts with the Na/K-ATPase

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Abstract

Depressed Ca-handling in cardiomyocytes is frequently attributed to impaired sarcoplasmic reticulum (SR) function in human and experimental heart failure. Phospholamban (PLN) is a key regulator of SR and cardiac function, and PLN mutations in humans have been associated with dilated cardiomyopathy (DCM). We previously reported the deletion of the highly conserved amino acid residue arginine 14 (nucleic acids 39, 40 and 41) in DCM patients. This basic amino acid is important in maintaining the upstream consensus sequence for PKA phosphorylation of Ser 16 in PLN. To assess the function of this mutant PLN, we introduced the PLN-R14Del in cardiac myocytes of the PLN null mouse. Transgenic lines expressing mutant PLN-R14Del at similar protein levels to wild types exhibited no inhibition of the initial rates of oxalate-facilitated SR Ca uptake compared to PLN-knockouts (PLN-KO). The contractile parameters and Ca-kinetics also remained highly stimulated in PLN-R14Del cardiomyocytes, similar to PLN-KO, and isoproterenol did not further stimulate these hyper-contractile basal parameters. Consistent with the lack of inhibition on SR Ca-transport and contractility, confocal microscopy indicated that the PLN-R14Del failed to co-localize with SERCA2a. Moreover, PLN-R14Del did not co-immunoprecipitate with SERCA2a (as did WT-PLN), but rather co-immunoprecipitated with the sarcolemmal Na/K-ATPase (NKA) and stimulated NKA activity. In addition, studies in HEK cells indicated significant fluorescence resonance energy transfer between PLN-R14Del-YFP and NKA α 1-CFP, but not with the NKA regulator phospholemman. Despite the enhanced cardiac function in PLN-R14Del hearts (as in PLN-knockouts), there was cardiac hypertrophy (unlike

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Disclosures

None

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PLN-KO) coupled with activation of Akt and the MAPK pathways. Thus, human PLN-R14Del is misrouted to the sarcolemma, in the absence of endogenous PLN, and alters NKA activity, leading to cardiac remodeling.

Keywords

Heart failure; Mutation; Calcium cycling; Phospholamban; SERCA

1. Introduction

Heart failure is a worldwide public health problem. Despite significant improvements in the management of heart failure symptoms, using therapies targeted to the neuro-hormonal axis with β -adrenergic receptor blockers and angiotensin-converting enzyme inhibitors, morbidity and mortality rates still remain high [1, 2]. Several signaling pathways have been implicated in the induction of cardiac disease and heart failure. However, the response of the heart to these diverse events indicates that only a few molecules are critical for instigating myocyte malfunction [3]. Among these, Ca is crucial for regulation of both cardiac excitation-contraction coupling and remodeling [4, 5]. In turn, Ca itself regulates and is regulated by the sarcoplasmic reticulum (SR) [6].

β -adrenergic stimulation of cardiac muscle initiates an important signal-transduction pathway [7], whereby elevation of cyclic AMP concentration activates PKA, which then phosphorylates several key proteins that affect overall cardiac function [8]. Among these proteins is phospholamban (PLN), which is a 52 amino acid SR membrane protein expressed abundantly in cardiac muscle. In its dephosphorylated form, PLN interacts with SERCA2a to inhibit Ca transport by lowering the apparent Ca affinity of SERCA2a [9, 10]. Upon phosphorylation of PLN, its inhibitory effect on SERCA2a is relieved and the SR Ca-store is increased [11, 12, 13]. The role of PLN in the regulation of basal contractility has been elucidated through the development of genetically engineered mouse models. PLN ablation (PLN-KO) significantly increases cardiac contractile parameters, whereas overexpressing PLN depresses cardiac function [14, 15]. The ability of PLN to regulate SERCA2a activity, thereby impacting the rate of cardiac relaxation and SR Ca-cycling, makes PLN a crucial regulator of cardiac function.

We previously identified a human PLN mutation in dilated cardiomyopathy patients entailing a deletion of amino acid arginine14 (R14) in the PLN gene coding region [16]. The patients with PLN-R14 deletion (PLN-R14Del) belong to a large Greek family with hereditary heart failure. Screening the family members did not yet reveal any homozygous individuals for the mutation. However, by middle age, heterozygous individuals developed left ventricular dilation, contractile dysfunction and episodic ventricular arrhythmias, with overt heart failure in some cases. In addition, a recent study on DCM patients of a German population identified an individual with the heterozygous PLN-R14Del mutation [17]. Pedigree analysis of the German family revealed that the PLN-R14Del mutation segregated with dilated cardiomyopathy and it was associated with cardiac death at early age, similar to our findings. Furthermore, all adult mutation carriers had attenuated R wave amplitudes on the standard ECG, irrespective of echocardiographic abnormalities, indicating that the mutation was involved in the cardiac remodeling process. To assess the functional significance of R14 deletion *in vivo*, we generated transgenic mice over-expressing the mutant human PLN-R14Del in the heart. These mice exhibited depressed cardiac function and recapitulated human cardiomyopathy, exhibiting similar histopathologic abnormalities and premature death as the human carriers [16].

To examine the effects of this mutant *in vivo* in the absence of PLN-WT (analogous to potential homozygous patients), we inserted the PLN-R14Del in the null (PLN-KO) mouse background. We report herein that the hyperdynamic contractility of PLN-KOs was not inhibited by the mutant PLN, although there was progression to cardiac hypertrophy. Further characterization indicated that the mutant PLN did not co-localize with SERCA2a, but it was misrouted to plasma membrane, interacting with and altering function of the Na/K-ATPase (NKA). Thus, in the absence of wild-type PLN, the Arg14 residue in PLN is critical for its insertion in the SR membrane and regulation of Ca-transport.

2. Materials and Methods

2.1 Generation of Transgenic Mice

Transgenic FVB/N mice with cardiac-specific expression of the murine PLN cDNA carrying the PLN-R14Del were mated with the phospholamban knockout (PLN-KO) mice (FVB/N). F₁ heterozygous PLN offspring with the PLN mutant transgene were identified, using PCR methodology and bred with PLN-KO mice to obtain F₂ pups. The PLN-KO offspring carrying the PLN mutant transgene were selected to backcross with PLN-KO mice for six generations before using them for our studies [11]. Handling and maintenance of animals was approved by the ethics committee of the University of Cincinnati. Eight- to 13-week-old mice were used for all studies.

2.2. Sarcoplasmic Reticulum Ca Uptake Rates and Immunoblot Assays in PLN-R14Del Hearts

To assess the levels of Ca-cycling proteins in wild type (PLN-WT), PLN-KO and PLN-R14Del mouse hearts, cardiac homogenates were subjected to quantitative immunoblotting [18, 19]. PLN levels were assessed using a monoclonal antibody (Millipore, USA; raised to purified bovine PLN) as well as a polyclonal antibody (Santa Cruz, USA). Calsequestrin was used as an internal control for protein loading. Oxalate-supported Ca-uptake in SR was also measured using cardiac homogenates by a modified Millipore filtration technique [20].

2.3. Left Ventricular Myocyte Measurements

Mouse left ventricular (LV) cardiomyocytes were isolated and mechanical properties and Ca transients were examined, as previously described [21]. Briefly, cell contraction was measured by video edge detection and intracellular-free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) was measured using Fura-2AM. Cells were perfused with normal Tyrode's (NT) solution (in mmol/L): NaCl 140, KCl 4, MgCl₂ 1, CaCl₂ 1, and HEPES 10, with pH 7.4, at 25°C. Twitches (steady state at 0.5 Hz) were field stimulated. To assess SR Ca content, 10 mmol/L caffeine was applied for 10 seconds in NT. Isoproterenol (100 nmol/L) was used to activate β -adrenergic signaling properties [20].

2.4. Immunofluorescence Staining

PLN-WT and PLN-R14Del hearts were fixed in paraformaldehyde, embedded in OCT (optimum cutting temperature) and sectioned as previously described [22]. For immunolocalization of proteins PLN monoclonal antibody (Millipore, USA) and SERCA2a polyclonal antibody (Bardilla, UK) were applied and subsequently visualized with goat anti mouse IgG/Alexa Fluor 488, and goat anti-rabbit IgG/Alexa 568 (Invitrogen, USA). Multiple images were acquired from 3 hearts/genotype, using a Nikon PCM 2000 confocal microscope equipped with argon and helium-neon lasers and Simple PCM software.

2.5. Histology

Mouse hearts were harvested, fixed overnight in 10% formalin, buffered with PBS, dehydrated in 70% ethanol, and transferred to xylene and then into paraffin. Paraffin-embedded heart samples were sectioned at 4 μ m and stained with Masson's Trichrome.

2.6. Na/K-ATPase Activity Measurements

Heart homogenates were prepared from PLN-WT and PLN-R14Del mice in homogenization buffer containing 0.315 mM sucrose, 1mM EDTA and 20mM Tris, pH 7.5. Na/K-ATPase activities were assessed with an enzyme-linked assay measuring the rate of ADP production, as linked to the rate of NADH fluorescence decrease in the absence or presence of 10 mM strophanthidin. Similar experiments were performed $\pm$ 20 mM KCl. Crude protein was incubated for 20 min at 37°C in a solution (pH 7.4) containing 100 mM NaCl, 8 mM MgCl₂, 40 mM Tris, 1 mM EGTA, 25 mM choline chloride, 1 U/mL lactate dehydrogenase, 1 U/mL pyruvate kinase, 1 mM phosphoenolpyruvate, and 80 μ M NADH. The reaction was initiated with the addition of 1 mM ATP. NADH fluorescence was continuously followed at excitation wavelength of 340 nm and emission wavelength of 460 nm for 20 min, using a spectrofluorometer (PTI Delta Scan-1; Photon Technology International, South Brunswick, NJ). Rates were calculated as μ M ATP hydrolyzed per hour per milligram of protein. Seven hearts (n = 7) from each model were used to determine NKA activity [23].

In addition, the same samples were assayed for K⁺-dependent para-nitrophenyl phosphatase (PNPPase) activity, as an additional test of NKA function. The reaction yielded *para*-nitrophenol, which is an intense yellow soluble product under alkaline conditions and was measured at 410 nm using a spectrophotometer.

2.7. Immunoprecipitation Experiments

Total PLN-WT and PLN-R14Del cardiac homogenates were solubilized at a protein concentration of 5 mg/ml in Buffer A [2% Triton, 20 mM Tris-Cl (pH, 7.4), 1 M NaCl, 1 mM dithiothreitol, 1 mM sodium vanadate, cocktail protease inhibitor tablet (Roche)] for 1 hour on a rotary wheel at 4°C. Solubilized proteins were obtained by centrifugation in a 50Ti rotor at 100,000 $\times$ g for 45 minutes at 4°C. The resulting supernatant was collected and diluted 10-fold in Buffer B [20 mM Tris-Cl (pH, 7.4), 1 mM dithiothreitol, 0.5% Tween, cocktail protease inhibitor tablet (Roche)]. Then, 1 mL of diluted solubilized sample was pre-cleared with 50 μ L protein G-agarose beads (Roche) overnight on a rotary wheel at 4°C. Protein G beads were precipitated, and the resulting pre-cleared supernatant or 1 mL Buffer B (negative control) was incubated with either anti-NKA α 1 isoform or anti-SERCA antibody overnight on a rotary wheel at 4°C. The resulting immunoprecipitates were then collected and washed 5 times (10 minutes each) in Buffer C [0.2% Triton, 20mM Tris-Cl (pH, 7.4), 0.15 M NaCl] on a rotary wheel at 4°C. Proteins were eluted from the antibody-Protein G bead conjugates by addition of SDS-sample buffer [60 mM Tris (pH, 6.8), 2% SDS, 10% glycerol, 100 mM dithiothreitol, 0.01% bromophenol blue] with vigorous mixing, and resolved using SDS gels. Immunoblots were probed with anti-NKA α 1 isoform, anti-SERCA, anti-PLN and anti-PLM-C2 (a gift from Dr. J. Cheung, Thomas Jefferson University, Philadelphia, Pennsylvania) antibodies.

2.8. Fluorescence Resonance Energy Transfer (FRET) Measurements

The NKA α 1-CFP, PLN-YFP and phospholemman (PLM)-YFP constructs were generated as previously described [24, 25]. The R14Del mutant of PLN was generated with the Quickchange Lightning mutagenesis kit (Stratagene) and custom oligonucleotide primers. The mutant was confirmed by sequencing. HEK-293 cells were cultured on glass coverslips prior to co-transfection of an excess (2- to 5-fold) of PLM-CFP and/or PLM-YFP, PLN-

WT-YFP and PLN-R14Del-YFP. For the FRET experiments assessing the interaction with NKA, HEK293 cells stably-transfected with NKA α 1-CFP were used. Fluorescence imaging was performed as previously described [25]. Briefly, FRET was measured using the acceptor photobleaching method. When FRET is present, the photobleaching of the acceptor (YFP) prevents FRET, with a consequent increase in the direct emission from the donor (CFP). Here, YFP was progressively photobleached for 10 min using 504/12 excitation. Images of CFP fluorescence (excitation 427/10, emission 472/30 nm) and YFP fluorescence (excitation 504/12 nm, emission 542/27 nm) were obtained every 10 seconds during the bleaching process. FRET efficiency was calculated from the fluorescence intensity of the CFP donor before (F_0) and after (F) acceptor-selective photobleaching, according to the following relationship: $E = 1 - (F_0/F)$.

2.9. Statistics

Data are presented as mean $\pm$ SEM. Statistical analysis was performed using linear regression and ANOVA and differences were considered significant if $P < 0.05$.

3. Results

3.1. Characterization of Transgenic Mice Expressing the Human PLN-R14Del Mutant

To assess the functional significance of the human PLN-R14Del mutant in the absence of the endogenous PLN gene, PLN-R14Del was introduced into PLN-KO mouse hearts. Three founder lines (1, 2, and 3), harboring the mutant PLN-R14Del transgene, were identified. Western blot analysis of cardiac homogenates from 3-month old transgenic mice revealed that the mutant PLN-R14Del protein levels in lines 1 and 2 were similar to the PLN-WT controls (Fig. 1(A and C)), while the protein levels in line 3 were 1.2 fold of the PLN-WT controls (data not shown). Further quantification of the PLN pentamer and monomer levels indicated no differences between transgenics and WTs (Fig. 1(A)). Lines 1 and 2 were then propagated for further characterization studies. Assessment of the SERCA protein levels in transgenic mouse hearts also showed similar expression to PLN-WT hearts (Fig. 1(A)). Since the amino acid residue R14 in PLN fulfills the requirement of a basic amino acid upstream for PKA-dependent phosphorylation at Ser 16 and its deletion might alter PLN phosphorylation, the degree of Ser16 phosphorylation was determined. The levels of Ser16 phosphorylation were significantly lower (7% of the PLN-WT) in PLN-R14Del transgenic hearts, compared with PLN-WTs (Fig. 1B). Furthermore, Thr17 PLN phosphorylation levels were undetectable in PLN-R14Del transgenic hearts vs. PLN-WTs (Fig. 1(B)). To avoid gender differences, characterization studies were carried out using male mice at 3 months of age.

3.2. Sarcoplasmic Reticulum Ca Uptake Rates in R14Del Hearts

To assess the functional consequence of the PLN-R14Del on the SERCA2a affinity for Ca, the initial rates of SR Ca uptake were measured in cardiac homogenates at different $[Ca^{2+}]$. The EC_{50} values in PLN-R14Del were lower ($0.170 \pm 0.010 \mu\text{mol/L}$) compared to PLN-WT control hearts ($0.294 \pm 0.008 \mu\text{mol/L}$) but similar to PLN-KO hearts ($0.143 \pm 0.020 \mu\text{mol/L}$). There was no significant difference in the maximal Ca-uptake velocity between the three groups (Fig. 1(D)). Since the EC_{50} in PLN-R14Del is similar to that in PLN-KO hearts, the mutant PLN-R14Del appears unable to functionally inhibit SERCA2a activity *in vivo*.

3.3. Mechanical Properties and Ca Transients in Isolated Cardiomyocytes

To elucidate whether mutant PLN-R14Del was associated with alterations in cardiac function, the contractile parameters of left ventricular (LV) cardiomyocytes were assessed. Under basal conditions, the fractional shortening (FS %) and the maximal rates of shortening ($+dL/dt$) and re-lengthening ($-dL/dt$) were similar between mutant PLN-R14Del

and PLN-KO cardiomyocytes. However, both mouse models exhibited significantly higher contractile parameters than WT controls, indicating that the PLN-R14Del function was hyper dynamic similar to the PLN-KO cardiomyocytes (Fig. 2(A, B and C)). To determine whether the hyper-contractile parameters could be stimulated by β -adrenergic agonists, PLN-mutant cardiomyocytes were subjected to maximal stimulation by isoproterenol (100 nM). Administration of isoproterenol did not further enhance the function of the PLN-R14Del or PLN-KO cardiomyocytes. Interestingly, the basal parameters in the mutant cardiomyocytes were similar to the maximally stimulated values in PLN-WT controls (Fig. 2(A, B and C)). However, the resting cell length was significantly increased in mutant, compared to PLN-WT cardiomyocytes, revealing the presence of cardiac remodeling in these mice (Fig. 2(D)).

To assess whether Ca transient properties mirrored the above contractile effects, mutant PLN-R14Del, PLN-KO and PLN-WT myocytes were loaded with 4 μ M Fura-2-AM to permit $[Ca]_i$ measurements. Under basal conditions, the peak amplitude of Ca transients in mutant PLN-R14Del and PLN-KO cells was significantly higher than PLN-WT control myocytes (Fig. 2(E)). Under isoproterenol stimulation, there was no further enhancement in the Ca transient amplitude of PLN-KO and PLN-R14Del cells, while PLN-WTs exhibited significant increases (Fig. 2(E)). The maximally stimulated parameters in PLN-WT cells were similar to those exhibited by mutant cardiomyocytes under basal conditions. In addition, SR Ca content was assessed by caffeine-induced Ca transients immediately after termination of steady state stimulation at 0.5 Hz. Without isoproterenol, the mean SR Ca content was similar in mutant PLN-R14Del and PLN-KO but it was significantly higher than WT controls (Fig. 2(F)). Isoproterenol increased SR Ca content significantly in PLN-WT control myocytes but had no further effects in mutant PLN-R14Del or PLN-KO cells (Fig. 2(F)). Thus, isoproterenol could not increase SR Ca-load, consistent with the hyperdynamic basal function in PLN-KO and PLN-R14Del cardiomyocytes.

3.4. Localization of the Mutant PLN in Cardiomyocytes

The lack of SERCA inhibition and functional effect of mutant PLN prompted us to determine whether PLN-R14Del was present along with SERCA in the SR, using confocal microscopy. Remarkably, while PLN colocalizes nicely with SERCA2a in WT mice (Fig 3, left) that was not the case in PLN-R14Del mice (Fig 3, right), despite similar expression levels of WT and mutant PLN.

3.5. Co-Immunoprecipitation Studies of Mutant PLN

The sarcolemmal Na/K-ATPase is modulated by phospholemman (PLM) in an analogous manner to SERCA regulation by PLN [26]. This raises the intriguing possibility that mutant PLN-R14Del is misdirected to sarcolemma and may interact with the sarcolemmal Na/K-ATPase. We tested this hypothesis by immunoprecipitating the $\alpha 1$ isoform of the Na/K-ATPase (NKA $\alpha 1$, the predominant cardiac isoform) and probing for the presence of PLN. We found that PLN-R14Del, but not PLN-WT, co-immunoprecipitated with NKA $\alpha 1$ (Fig. 4(A)). We then also probed the immunoprecipitations for the presence of the Na/K-ATPase regulatory protein phospholemman (PLM), using the PLM-C2 antibody. PLM was indeed present in the NKA immunoprecipitates of both PLN-WT and PLN-mutant hearts (Fig. 4(A)). Further experiments, using the anti-SERCA2a antibody, confirmed that PLN co-immunoprecipitated with its interacting partner SERCA2a from WT hearts. However, SERCA2a failed to co-immunoprecipitate PLN from the PLN-R14del mutant hearts (Fig. 4(B)). In regard to NKA, there was no association between NKA and SERCA in either WT or mutant hearts (Fig. 4(B)). Nonimmune IgG was used as a control for the immunoprecipitation studies, which failed to pull down any of the proteins (data not shown).

3.6. FRET Measurements of the PLN-R14Del - NKA Interaction

To further test whether and how the PLN mutant interacts with NKA or with its regulator phospholemman (PLM or FXYD-1), we employed FRET assays in HEK cells. PLN-WT-YFP is largely confined to intracellular membranes (Fig. 5(A) bottom left panel), but PLN-R14Del-YFP is clearly apparent at the plasma membrane (Fig. 5(A) bottom right panels). Moreover, plasma membrane-targeted PLN-R14Del-YFP co-localized with NKA α 1-CFP (upper panels) and their interaction resulted in measurable FRET. Upon acceptor photobleaching, donor fluorescence ($F_{\text{NKA}\alpha 1\text{CFP}}$) increased by $12.5\% \pm 0.03$ in cells, where sufficient PLN-R14Del-YFP was present to saturate NKA-CFP. In contrast, no FRET was observed for WT-PLN-YFP (slight donor photobleach was present instead; Fig. 5(B)). In parallel experiments, NKA α 1-CFP FRET with PLM-YFP resulted in a $F_{\text{NKA}\alpha 1\text{CFP}}$ increase by $18.0\% \pm 0.03$. Thus, when PLN-R14del is misdirected to the plasma membrane it can interact with NKA in a manner that mimics to some extent the natural sarcolemmal NKA-PLM interaction.

Like PLN, PLM is known to form homo-multimers in the plasma membrane, so we tested for FRET between PLM-CFP and PLN-R14del-YFP (Fig. 5(C)). While there was detectable FRET (<5%), this was far less than the previously described robust PLM-PLM FRET [25] (nearly 40% measured here). Thus, our data suggest that PLN-R14del-YFP interacts directly with the Na/K-ATPase molecule, but does not multimerize with PLM. Notably, mutant PLN-R14Del can still form homopentamers (Fig. 1(A)).

3.7. Na/K-ATPase Activity in PLN-R14Del Mutants

Based on these findings, we tested whether the mutant PLN could affect NKA activity. We assessed NKA activity using the ability of 20 mM K^+ to stimulate NKA (Fig. 6). This enzymatic activity was significantly increased in the PLN-R14Del compared with PLN-WT hearts (Fig. 6(A)), suggesting modulation of the NKA by the mutant PLN-R14Del. These findings were further validated by performing the colorimetric PNPP assay, which measured K^+ stimulated PNPPase activity (Fig. 6(B)). Importantly, the NKA expression levels were similar between PLN-R14Del and PLN-WT (Fig. 6(C)). This suggests that interaction with PLN-R14Del may increase NKA activity.

Furthermore, we assessed NKA activity using a specific inhibitor, strophanthidin (Fig. 7). In the presence of strophanthidin, the NKA-independent ATPase activity was similar between WT and PLN-R14Del cardiac homogenates (Fig. 7(A)), suggesting that the higher ATPase activity in mutant hearts in the absence of strophanthidin was due to NKA. Indeed, the strophanthidin-sensitive NKA activity was increased by ~2.5-fold in the PLN-R14Del compared to PLN-WT hearts (Fig. 7(B)), in agreement with the K -stimulated PNPPase activity (Fig. 6(A)). Remarkably, ouabain (which normally inhibits NKA) greatly stimulated the NKA activity only in PLN-R14Del heart homogenates. This suggests that the misrouted PLN might alter the how ouabain alters NKA function (supplemental Fig. 1).

Since NKA activity was increased, we tested whether sodium calcium exchanger (NCX) expression may be altered. The expression levels of the NCX protein were significantly increased in PLN-R14Del compared with PLN-WT (Fig. 6(D)). However, there was no observed association between the PLN-R14Del and NCX in immunoprecipitation studies.

3.8. Cardiac Remodeling and Histology

Since resting cell length (Fig. 2(D)) indicated the presence of remodeling in mutant PLN-R14Del hearts, gravimetric analysis of heart and body weights was pursued in 3 month-old mice. Expression of the mutant PLN-R14Del was associated with increases in heart to body weight ratios as well as lung to body weight ratios, compared with PLN-WT controls (Fig.

8(A and B)). M-mode echocardiography [27] also revealed increased wall thickness in mutant compared with WT hearts (LVPWd 1.77 ± 0.08 mm vs. 1.33 ± 0.08 mm; $p=0.001$ and LVPWs 2.04 ± 0.08 mm. vs. 1.76 ± 0.09 mm; $p=0.02$, respectively). Furthermore, there was a decrease in the LV volume in PLN-R14Del hearts as compared with the PLN-WT (diastole 37.3 ± 4.6 μ l vs. 54 ± 6.5 μ l; $p=0.02$ and systole 10.1 ± 1.6 μ l vs 13.9 ± 0.7 μ l; $p=0.03$). The increases in heart/body weight were associated with induction of a fetal gene program with specific increases of β -myosin heavy chain isoform (β -MHC) protein levels (data not shown), indicating pathological alterations in the mutant phospholamban transgenic hearts. The observed remodeling is not likely associated with the increased Ca-cycling in mutant hearts, since previous studies showed that similarly enhanced function in PLN deficient hearts did not result in any morphological or histological alterations [28]. The hypertrophic gene expression changes in R14Del-PLN hearts may be associated with increased expression of NCX, as previously suggested [29, 30].

To further assess the hypertrophic and pathological alterations in the mutant PLN hearts, histologic analysis was performed at 3 months of age. The PLN-R14Del mouse hearts showed a dramatic increase in size relative to PLN-WT controls (Fig. 8(C)). Furthermore, histological analysis revealed ventricular dilation, myocyte disarray and myocardial interstitial fibrosis in transgenic hearts compared to their PLN-WT counterparts (Fig. 8(D)).

3.9. Signaling Pathways in PLN-R14Del Hearts

The presence of cardiac remodeling in 3 month old PLN-R14Del mice prompted us to investigate activation of survival or hypertrophic signaling pathways in this model. Since the mitogen-activated protein (MAP) kinase family has been implicated in different aspects of cardiac regulation, from development to pathological remodeling, we selected to examine the MAP kinase (ERK1/2, JNK, P38) as well as the PI3K/AKT pathways. The results showed increases of 27% in total P38 and 30% in phosphorylated P38 protein levels, compared with the PLN-WT hearts. While the ERK1/2 total protein levels remained unchanged, the phosphorylated ERK level was increased by 15%, compared to PLN-WT. Similarly, the JNK pathway did not reveal any increases in the total protein but the phosphorylated JNK was higher (25%) in PLN-R14Del compared to PLN-WT. In addition, there was no change in total AKT protein levels, while 25% increase was observed in phosphorylated AKT, compared with WT hearts. Overall, the observed alterations in these players were modest but increases in the activity of MAP kinase pathway players could have contributed to the hypertrophic response, while activation of AKT could have contributed to the stability of cardiac cells, consistent with previous observations with other hypertrophic models [31, 32].

4. Discussion

This is the first study to demonstrate that a human mutation in the PLN coding region, may lead to mis-localization of PLN from SR to sarcolemma and alter NKA activity. The PLN mutant with Arginine 14 deletion is associated with inherited human dilated cardiomyopathy and premature death [16] in the heterozygous state. Indeed co-expression of wild-type PLN along with the mutant-PLN targeted PLN-R14Del to the ER/SR in HEK cells and resulted in superinhibition of SERCA2 activity [16]. However, when this mutant was introduced into the PLN null background, there was no effect on SERCA2a or cardiac function, as if these were PLN null mice, although the hearts presented with hypertrophy, interstitial fibrosis and myocyte disarray by 12 weeks of age. Further confocal microscopy studies revealed that the PLN-R14Del mutant was not colocalized with SERCA2a in the SR membranes. A disrupted association between PLN and SERCA2a would be consistent with the inability of isoproterenol to further enhance the contractile parameters in PLN-R14Del mice. Thus, the presence of endogenous wild type PLN may correct the mislocalization of mutant PLN.

However, insertion of mutant PLN in the null background, representing the homozygous R14Del-PLN patients, may provide a better model to understand the mechanisms associated with the effects of this mutant by itself in the heart.

Of note, the mutant PLN was misrouted to plasma membrane where it interacted with NKA, causing an increase in NKA enzymatic activity. This is the first report that PLN can interact with NKA in the cardiac myocyte and modulate NKA function. This interaction was substantiated by the fact that NKA and PLN could form a protein-protein complex, revealed by co-immunoprecipitation studies and confirmed by FRET measurements.

The mechanisms underlying the mis-localization of mutant PLN may involve disruption of the N-terminal Di-Arginine motif, which has been recently shown to be important in targeting PLN to ER/SR [33]. This study further emphasizes that the R14 residue in PLN, which is highly conserved among species, is important for PLN localization to the SR membrane, impacting SR Ca-handling and cardiac contractility *in vivo*. In regard to PLN trafficking, Stenoien et al [34] reported that PLN is present in highly mobile vesicular structures outside the endoplasmic reticulum in skeletal myoblasts and concluded that PLN targeting was regulated through vesicle trafficking. They suggested that PLN was trafficked through directed transport via the golgi to the plasma membrane before endosome-mediated internalization. Thus, the observed plasma membrane localization of our mutant PLN indicates that it may not be able to follow the classical trafficking pathway ending with endoplasmic-mediated internalization. Consequently, mutant PLN remains localized to the plasma membrane and does not traffic to the SR.

It is intriguing that sarcolemma-localized mutant PLN interacted with NKA, which is known to be regulated by PLM. PLM and PLN have similar functional regulatory properties: both are small transmembrane proteins that interact with P-type ATPases [35] and both have PKA consensus phosphorylation motifs [36]. Both PLN and PLM serve as tonic inhibitors of SERCA and NKA respectively, such that phosphorylation or removal of PLN or PLM enhances the $[Ca]_i$ or $[Na]_i$ affinity of the pumps. In addition, SERCA and NKA present high amino acid and structural homology [37] and we hypothesized that misrouted PLN could interact with NKA similar to PLM, resulting in our observed effects on NKA activity. Somewhat surprising is that PLN-R14del activated rather than inhibited NKA. Sequence alignment between SERCA and NKA did not reveal any shared binding sites for PLN or PLM. Thus, PLN may affect NKA activity directly or indirectly by influencing the PLM/NKA complex. In addition, we observed that mutant PLN interfered with the ability of ouabain to inhibit NKA activity in the transgenic hearts (converting ouabain to an NKA activator). Endogenous cardiac glycosides have been shown to mediate signal transduction through NKA resulting in cellular differentiation and proliferation [38]. Therefore, the PLN-R14Del interaction with NKA may alter the signaling scaffold, thus affecting hypertrophic signaling [39].

Although we observed enhanced Ca cycling and contractility in the PLN-R14Del mice, similar to the PLN-KO, the mutant-PLN hearts presented with cardiac hypertrophy at 3 months of age. This is not likely due to the enhanced calcium cycling, since the PLN-KO does not present any geometrical abnormalities (28) or alterations in the expression levels of calcium cycling proteins even up to 1 year of age (40). Thus, it is tempting to speculate that the sarcolemmal misdirection and interaction with NKA might be involved in the hypertrophic effect of PLN-R14del (as suggested above). Indeed, extensive data have suggested that Na/K-ATPase serves as a mediator in cell signaling via interaction with the cardiac glycoside, ouabain [39], which may trigger hypertrophic responses. NKA can act as a signal transducer, resulting in activation of multiple signal transduction pathways such as Src kinase, transactivation of the epidermal growth factor receptor by Src, activation of Ras

and p42/44 mitogen-activated protein kinases, and increased generation of reactive oxygen species by mitochondria in epithelial and cardiac cells [39, 41, 42, 43]. In cardiac myocytes, these downstream events have been shown to result in the induction and regulation of a number of cardiac growth-related genes, and myocyte hypertrophy [41, 42, 43, 44, 45, 46]. Our findings suggest that multiple signaling circuits, including MAP kinase pathways, were activated and may have contributed to hypertrophy in PLN-R14Del hearts. Furthermore, up regulation of the AKT pathway may have increased the stability of cardiac cells in this model [31, 32]. Nevertheless, this observed remodeling may present a limitation in the present study, as it may impact the phenotype and the obtained alterations in NKA activity by PLN-R14Del.

In summary, our findings point to a primary defect in proper localization of the human PLN-R14Del to the SR membrane *in vivo*. The mutant PLN is misrouted to the sarcolemma and associates with NKA, enhancing its enzymatic activity. In this respect, regulation of NKA by the PLN-R14Del is opposite to the regulation by the endogenous NKA regulator PLM, which inhibits NKA activity unless phosphorylated. Future studies on the cellular and molecular mechanisms in this model, using gene expression profiling and proteomics, may unveil specific molecules or pathways by which the human PLN mutant influences cardiac function and remodeling. These studies may also provide further insights into potential therapeutic targets in homozygous patients carrying this mutant PLN.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SR	sarcoplasmic reticulum
SERCA2a	Cardiac sarcoplasmic reticulum Ca ²⁺ -ATPase
PLN	phospholamban
βAR	β-adrenergic receptor
DCM	dilated cardiomyopathy
NKA	Na/K ATPase

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Fig. 1.

Quantitative immunoblotting of PLN and SERCA2a in mouse cardiac homogenates and SR Ca uptake in transgenic and wild type hearts. (A): Cardiac homogenates were subjected to 13% SDS-polyacrylamide gel electrophoresis and electroblotted on nitrocellulose membranes. The membranes were probed with a PLN antibody or SERCA antibody. PLN_p, PLN pentamer; PLN_m, PLN monomer. (B): Expression levels of phosphorylated PLN protein at Ser¹⁶ and Thr¹⁷ in PLN-R14Del relative to PLN-WT hearts. (C): Quantification of total PLN protein in PLN-R14Del (Lines 1 and 2) relative to PLN-WT hearts. Data represent the mean $\pm$ SEM. A minimum of 4 hearts were analyzed for each genotype or line. (D): Initial rates of SR Ca uptake in cardiac homogenates from PLN-WT (●, $n = 4$), PLN-KO (■, $n = 4$) and PLN-R14Del (▲, $n = 4$) mice. The points represent the mean $\pm$ SEM of four experiments (each in triplicate), using individual hearts. The data were analyzed by nonlinear regression using Microcal Origin software (version 6.0). Inset shows the EC₅₀ values for PLN-WT ($n = 4$), PLN-KO ($n = 4$) and PLN-R14Del ($n = 4$) hearts. The maximal Ca-uptake velocities (V_{max}) were 63.4 ± 3.8 mol/mg/min for PLN-WT, 81.1 ± 9.9 mol/mg/min for PLN-KO and 74.2 ± 10.0 mol/mg/min for PLN-R14Del.

Fig. 2. Mechanical parameters and Ca transients in isolated cardiomyocytes at baseline and under isoproterenol (100nM) stimulation. (A): Rates of shortening (+dL/dt), (B): Rates of relengthening (-dL/dt), (C): Shortening fraction (FS%), and (D): Cell length in isolated cardiomyocytes from wild type mice (PLN-WT, $n = 5$), PLN-KO ($n = 4$) and mutant PLN mice (PLN-R14Del, $n = 6$). Values are mean $\pm$ SEM. At least 27 to 80 cells were used for measurements of each parameter. *, $p < 0.05$ vs PLN-WT. (E): Ca transient amplitude (340/380 nm ratio) obtained from PLN-WT ($n = 4$), PLN-KO ($n = 4$) and PLN-R14Del ($n = 4$). Values are mean $\pm$ SEM. A minimum of 42–70 cells were studied. *, $p < 0.05$ vs PLN-WT. (F): Analysis of caffeine-induced Ca transients in PLN-WT ($n = 7-9$), PLN-KO ($n = 4$) and PLN-R14Del ($n = 4$) cardiomyocytes. 10 mmol/L caffeine was applied and caffeine induced amplitude was assessed. Values are mean $\pm$ SEM. A minimum of 30 cells were studied. *, $p < 0.05$ vs PLN-WT.

Fig. 3. Localization of PLN and SERCA2a proteins in PLN-WT and PLN-R14Del mouse hearts, using immunofluorescence staining. Heart sections from 3 month-old mice were stained with SERCA2a antibody (red) and counterstained with PLN antibody (green). Confocal microscopy was used to assess SERCA2a and PLN localization in wild type (PLN-WT) or mutant PLN (PLN-R14Del) hearts. MERGE indicates the overlay of images. Insets show a higher magnification of the cardiac cell.

Fig. 4. Immunoprecipitations (IP) with either NKA or SERCA antibodies. (A): the NKA antibody was used for immunoprecipitations and blots were probed with NKA (upper panel), PLN (middle panel) and PLM (lower panel) antibodies. All the samples were analyzed on the same gel. (B): upper panel, the SERCA2 specific antibody was used for immunoprecipitation; middle panel, the NKA α 1 isoform specific antibody was used as a probe for NKA detection; and lower panel, the PLN specific antibody was used to detect PLN.

Fig. 5.

Fluorescence resonance energy transfer (FRET) measurement of PLN-R14Del and NKA interaction. (A): HEK293 cells stably expressing NKAα1-CFP (upper panels) were transfected with YFP-tagged wild-type PLN (PLN-YFP, bottom left) or mutant PLN (PLN-R14Del-YFP, bottom right). (B): Average increase of donor (NKA-CFP) fluorescence after YFP photobleach (normalized to prebleach), indicative of FRET between NKA and PLN-R14Del, but not PLN WT. Controls: Ctl is untransfected and YFP-tagged PLN and phospholemman (PLM) transfected cells were negative and positive controls, respectively. (C): Average increase of donor fluorescence (PLM-CFP) after photobleach (normalized to prebleach), indicative of FRET between PLM-PLM but only minor FRET between PLM-PLN-R14Del. At least 3 separate experiments were performed and values are mean ± SEM, where * indicates $p < 0.05$.

Fig. 6.

Assessment of NKA activity and expression in PLN-WT and PLN-R14Del hearts. (A): Total K^+ sensitive ATPase activity was increased in PLN-R14Del hearts relative to PLN-WT hearts. $n = 8$ (B): K^+ stimulated PNPP assay, indicating significant NKA enzymatic activity in PLN-R14Del hearts. $n = 6$ (C): Measurement of the NKA expression levels. $n = 8$ (D): Analysis of sodium calcium exchanger (NCX) expression levels in PLN-WT and PLN-R14Del hearts. $n = 4$. Values are mean $\pm$ SEM. *, $p < 0.05$ vs PLN-WT.

Fig. 7.

Strophanthidin-sensitive NKA activity in PLN-WT and PLN-R14Del hearts. (A): Total ATPase activity was increased in R14del cardiac homogenates under basal conditions or in the absence of an inhibitor. ATPase activities in the presence of strophanthidin, a specific NKA inhibitor, were similar between PLN-WT and PLN-R14Del hearts, suggesting that the increase in basal total ATPase activity was not due to the contribution of other ATPases but an increase in NKA. (B): NKA activity was calculated by subtracting the ATPase activity in the presence of inhibitor from the activity in the absence of inhibitor. The average of NKA activity in PLN-R14Del heart homogenates was expressed as a fold-increase over PLN-WT for each paired sample. $n=7$. *, $p<0.05$.

Fig. 8. Gravimetric and histologic analyses of wild type (PLN- WT) and mutant PLN-(PLN-R14Del) mice. (A and B): Heart, lung and body weights were measured from PLN-WT and PLN-R14Del mice. $n = 8$. Values are mean $\pm$ SEM. *, $p < 0.05$ vs PLN-WT. (C): Images of hearts from PLN-WT and PLN-R14Del mice at 3 months of age. (D): Longitudinal sections of hearts stained with Masson's trichrome to reveal interstitial tissue fibrosis.