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Effects of naturally occurring arginine 14 deletion on phospholamban conformational dynamics and membrane interactions☆,☆☆

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ABSTRACT

Phospholamban (PLN) is a single-pass membrane protein that regulates the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA). Phosphorylation of PLN at Ser16 reverses its inhibitory function under β-adrenergic stimulation, augmenting Ca^{2+} uptake in the sarcoplasmic reticulum and muscle contractility. PLN exists in two conformations; a T state, where the cytoplasmic domain is helical and adsorbed on the membrane surface, and an R state, where the cytoplasmic domain is unfolded and membrane detached. Previous studies have shown that the PLN conformational equilibrium is crucial to SERCA regulation. Here, we used a combination of solution and solid-state NMR to compare the structural topology and conformational dynamics of monomeric PLN (PLN^{AFA}) with that of the PLN^{R14del}, a naturally occurring deletion mutant that is linked to the progression of dilated cardiomyopathy. We found that the behavior of the inhibitory transmembrane domain of PLN^{R14del} is similar to that of the native sequence. Conversely, the conformational dynamics of R14del both in micelles and lipid membranes are enhanced. We conclude that the deletion of Arg14 in the cytoplasmic region weakens the interactions with the membrane and shifts the conformational equilibrium of PLN toward the disordered R state. This conformational transition is correlated with the loss-of-function character of this mutant and is corroborated by SERCA's activity assays. These findings support our hypothesis that SERCA function is fine-tuned by PLN conformational dynamics and begin to explain the aberrant regulation of SERCA by the R14del mutant. This article is part of a Special Issue entitled: NMR Spectroscopy for Atomistic Views of Biomembranes and Cell Surfaces. Guest Editors: Lynette Cegelski and David P. Weliky.

1. Introduction

Calcium transport in the heart muscle is orchestrated by several different membrane proteins. In particular, the uptake of Ca^{2+} ions into the sarcoplasmic reticulum (SR) is governed by a membrane protein complex between the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) and phospholamban (PLN) [1–3]. This complex is responsible for about 70% of the Ca^{2+} transport into the SR in humans. The paramount importance of the SERCA/PLN complex stems from its direct involvement in the cardiac output [4]. Dysfunctions in Ca^{2+} handling by this membrane protein complex lead to the progression of several cardiomyopathies with eventual development of heart failure [5]. To date, several different mutations of the *pln* gene have been sequenced and identified in humans who develop hereditary cardiomyopathies. Among those, a mutation in the *pln* promoter, a truncation resulting in a $PLN^{L39stop}$ mutant, aberrant R9C, R9L, and R9H mutations, as well as *pln* gene duplications, have been directly linked to either dilated or hypertrophic cardiomyopathy [6–9]. Additionally, a recurring deletion of arginine 14 (R14del) in the regulatory domain of PLN was first observed only in heterozygous patients suffering from dilated cardiomyopathy (DCM), a disorder of the heart manifested as enlargement of the left ventricle [8]. Subsequent genetic screening studies of a wider population, broadened the impact of this PLN mutant, not only identifying the Arg14 deletion in patients diagnosed with dilated cardiomyopathy, but also in those with arrhythmogenic right ventricular cardiomyopathy [10].

Powered by ATP, SERCA translocates two Ca^{2+} ions into the SR in exchange for three H_3O^+ ions. It is reversibly inhibited by PLN, a 52 amino acid single-pass membrane protein [11]. PLN exists as a pinwheel-

Abbreviations: DCM, dilated cardiomyopathy; LOF, loss of function; PKA, protein kinase A; PLN^{AFA}, phospholamban monomer bearing mutations C36A C41F C46A; PLN^{WT}, wild type phospholamban pentamer; pSer, phosphoserine; SERCA, sarco(endo)plasmic reticulum Ca^{2+} ATPase; R14del, phospholamban mutant lacking arginine in position 14; SE-PISEMA, sensitivity enhanced polarization inversion spin exchange at magic angle; SR, sarcoplasmic reticulum; TM, transmembrane

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shaped pentamer, with the inhibitory region comprised of the transmembrane domain II and more hydrophilic domain Ib crossing the lipid membrane with a ~15–20° tilt angle [12–14]. The transmembrane domains of the protomers are arranged in a left-handed coiled-coil of approximately 40 Å in length. In the ground state, the cytoplasmic, regulatory region (loop and domain Ia) is adsorbed on the membrane surface. This interaction is stabilized by the energetically favorable contacts between the aliphatic residues of the domain Ia helix with the hydrophobic core of the membrane, as well as the electrostatic interactions of the polar residues with the lipid head groups and the bulk solvent. Mutagenesis and biophysical data have lead to a regulatory model in which de-oligomerized PLN forms a 1:1 inhibitory complex with the ATPase [11,15]. Following phosphorylation at Ser16 by protein kinase A (PKA), PLN inhibition is negated and Ca^{2+} flux in the SR is augmented. This model was recently confirmed by three different crystal structures obtained with PLN and its homologous analog sarcolipin (SLN) in complex with SERCA [16–18]. The inhibitory domains of the two proteins (i.e., the transmembrane domain II for PLN) bind in a hydrophobic groove between TM2 and TM9 of the ATPase. However, the scarce resolution of electron density for the cytoplasmic domain of PLN did not enable the clarification of the molecular details of its regulatory mechanism.

In the past years we have used NMR spectroscopy to determine PLN structures free and bound to SERCA [13,19,20]. Based on our studies, we proposed a regulatory model in which PLN conformational equilibrium is central to SERCA regulation. We found that PLN exists in three main states: a ground state (T state) with the cytoplasmic domain adsorbed on the surface of the lipid membrane, an excited state (R state), where the cytoplasmic domain is unfolded and membrane detached, and a SERCA bound state (B state), where the transmembrane domain of PLN is bound to SERCA and the extended domain Ia interacts with SERCA's cytoplasmic domains [21–23]. Phosphorylation at Ser16 shifts the equilibrium toward the B state with a local structural rearrangement [19]. The existence of the different PLN structural states is corroborated by mutagenesis studies, demonstrating that it is possible to promote the R and B states with concomitant relief of SERCA inhibition [24].

Here, we investigated how the alteration of the conserved amphipathic motif via a naturally occurring amino acid deletion in the cytoplasmic domain affects PLN conformational equilibrium and its regulation of SERCA. Challenges associated with studying membrane proteins called for a combination of solution and solid-state NMR experiments [25]. We found that the deletion of Arg14 slightly alters the transmembrane domain of the protein, as well as induces a pronounced shift of the conformational equilibrium of the cytoplasmic domain toward the membrane-detached R state. Furthermore, we found that in contrast with PLN, synthetic phosphorylation of PLN^{R14del} does not relieve inhibition of SERCA. This suggests that the PLN^{R14del} mutant functions as a constant inhibitor of SERCA, unresponsive to typical regulatory mechanisms like β-adrenergic stimulation. Analyzing these data with the dynamic ruler we developed for site specific mutants of PLN, our data begins to rationalize the loss-of-function nature of R14del and how it influences not only Ca^{2+} transport in the SR but also the development of cardiomyopathies for individuals carrying this mutation.

2. Material and methods

2.1. Protein production

Phospholamban with a deletion of Arg14 was produced from PLN^{AFA} by site-directed mutagenesis, using QuikChange kit (Stratagene, La Jolla, CA). Expression and purification steps were identical to the host sequence [26]. Briefly, the construct was expressed with R14del fused with maltose binding protein via a tobacco etch virus protease cleavage site. Fusion protein was purified by affinity chromatography on amylose resin, and upon cleavage — by high performance liquid chromatography. To produce Ser16 phosphorylated R14del for the activity assays, we have employed microwave assisted solid phase peptide synthesis as described previously, using Fmoc-Ser[PO(OBzl)] (Merck, Darmstadt, Germany). Phosphoserine was coupled at 50 °C; subsequent deprotection steps were performed at room temperature and amino acids 1–15 were coupled at 50 °C to reduce the risk of dephosphorylation. SDS-PAGE of Ser16-phosphorylated R14del is shown in Supplementary Fig. 1.

2.2. Nuclear magnetic resonance

Solution NMR was performed as described previously. Phospholamban was reconstituted in dodecylphosphocholine-d_{38} and the relaxation measurements were performed at 600 MHz proton frequency on a Varian spectrometer [27]. Separated local field experiments were performed on R14del reconstituted in DMPC:POPC 4:1 bicelles, doped with 1% (mol) DMPE-PEG350 and 1% (mol) PE-DTPA to promote ytterbium ion chelation. Protein was reconstituted in long chained lipids, followed by the addition of the capping lipid (1,2-dihexanoyl-*sn*-glycero-3-phosphocholine; DHPC) [28]. The q-ratio (total long chained lipid to short chained lipid) was 4.5. To promote the parallel orientation of the bicelles $YbCl_3$ was added to the final concentration of 8 mM [29]. The ^{15}N–^{1}H SE-PISEMA experiment was performed on uniformly labeled ^{15}N PLN at 700 MHz on a Varian spectrometer. Magic angle spinning experiments were recorded on uniformly labeled $^{13}C/^{15}N$ PLN in DMPC-d_{54}. Multilamellar vesicles containing PLN were pelleted down overnight at 250,000 g; the pellet was transferred into a Bruker 22 μl rotor and additional buffer was added to ensure that the sample retains hydration throughout the experiment. Refocused INEPT ^{13}C–^{1}H spectra were recorded at 10 kHz spinning rate on a 700 MHz spectrometer.

2.3. SERCA ATPase assays

SERCA1a was extracted from rabbit skeletal muscle and purified using affinity chromatography using previously described methods [30]. SERCA1a and PLN were co-reconstituted in 1,2-dioleoyl-*sn*-glycero-3-phosphocholine: 1,2-dioleoyl-*sn*-phosphoethanolamine (Avanti, Alabaster, AL) at a 4:1 molar ratio in lipid vesicles as previously described [31]. Molar ratios of 10:1 PLN:SERCA and 700:1 lipids:SERCA were used. The Ca^{2+} dependence of SERCA's ATPase activity was measured spectrophotometrically with a Spectromax microplate reader (Molecular Devices) as a function of NADH consumption using coupled enzyme assays at 37 °C as previously described [32,33]. Solutions were equilibrated at 37 °C before the experiment and reaction plate was incubated at 37 °C for 20 min before reaction initiation. The initial rate of ATPase activity (V) was measured as a function of calcium concentration and the data were fit using a standard Hill equation:

$$V = \frac{V_{max}}{1 + 10^{n(pK_{Ca} - pCa)}}$$

where V is the initial rate of ATPase activity, V_{max} is the maximal rate, n is the Hill coefficient, pCa is the log of the Ca^{2+} concentration, and pK_{Ca} is the pCa value where $V = V_{max} / 2$.

The assays were performed with three separate reconstitutions, with triplicate measurements for each separate reconstitution.

3. Results

3.1. SERCA ATPase assays

Haghighi and coworkers found that PLN^{R14del} acted as a partial loss-of-function mutant in microsomal preparations [8]. In this study, we measured the effect of the R14del mutant of PLN on SERCA activity for both unphosphorylated and phosphorylated PLN^{R14del} in reconstituted lipid vesicles. We found that the ΔpK_{Ca} for PLN^{R14del} is 0.17, which indicates a loss of inhibitory potency with respect to PLN^{AFA} ($\Delta pK_{Ca} = 0.26$)