Reversal of Phospholamban Inhibition of the Sarco(endo)plasmic Reticulum Ca\(^{2+}\)-ATPase (SERCA) Using Short, Protein-interacting RNAs and Oligonucleotide Analogs*

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Kailey J. Soller†, Jing Yang‡, Gianluigi Veglia§†, and Michael T. Bowser†‡
From the Departments of †Chemistry and ‡Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Minneapolis, Minnesota 55455

The sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) and phospholamban (PLN) complex regulates heart relaxation through its removal of cytosolic Ca\(^{2+}\) during diastole. Dysfunction of this complex has been related to many heart disorders and is therefore a key pharmacological target. There are currently no therapeutics that directly target either SERCA or PLN. It has been previously reported that single-stranded DNA binds PLN with strong affinity and relieves inhibition of SERCA in a length-dependent manner. In the current article, we demonstrate that RNAs and single-stranded oligonucleotide analogs, or xeno nucleic acids (XNAs), also bind PLN strongly (\(K_d < 10\) nM) and relieve inhibition of SERCA. Affinity for PLN is sequence-independent. Relief of PLN inhibition is length-dependent, allowing SERCA activity to be restored incrementally. The improved in vivo stability of XNAs offers more realistic pharmacological potential than DNA or RNA. We also found that microRNAs (miRNAs) 1 and 21 bind PLN strongly and relieve PLN inhibition of SERCA to a greater extent than a similar length random sequence RNA mixture. This may suggest that miR-1 and miR-21 have evolved to contain distinct sequence elements that are more effective at relieving PLN inhibition than random sequences.

Calcium cycling in cardiomyocytes is a tightly regulated process that ensures proper muscle contractility (1, 2). Many of the proteins involved in this cycle have been implicated in cardiac failure, the leading cause of death worldwide (3, 4). Given the complexity of heart failure phenotypes, strategies for reversing declining cardiac performance are diverse and necessary. Gene therapy efforts for inherited forms of heart disease have been complicated by the high apparent calcium affinity and hampering Ca\(^{2+}\) transport of SERCA (11). Upon \(\beta\)-adrenergic stimulation, protein kinase A (PKA) will phosphorylate PLN at Ser-16, restoring the apparent calcium affinity and Ca\(^{2+}\) transport of SERCA (11).

We previously reported that random sequence, single-stranded DNA and RNA bind to PLN with high affinity (\(K_d < 10\) nM), and more importantly, relieve its inhibitory effects on SERCA, restoring the enzyme’s basal activity. We found that PLN remained bound to SERCA upon the addition of ssDNA, and that the effects of ssDNA on the SERCA-PLN complex are length-dependent and tunable (12). We propose to refer to these unique sequences as short, protein-interacting DNAs and RNAs (i.e. SPIDRs).

In the current study, we probe the chemical nature behind this unexpected interaction and the reversal of SERCA inhibition. In addition, although ssDNA and RNA are readily degraded in vivo, the chemically modified oligonucleotide analogs, or XNAs, chosen in this work are nuclease-resistant and are therefore much better scaffold candidates for drug development.

Secondly, we examine two specific microRNAs (miRNAs), determining both how strongly they bind PLN and their functional effects on the SERCA-PLN complex. Non-coding...
miRNAs play central roles in gene regulation and pathology (13). In the heart, miRNAs are involved both in cardiogenesis (14, 15) and in disease including: diabetic cardiomyopathy, hypertrophy, ischemia, and electrical remodeling (16–22). Recent developments in both therapeutic inhibition and enhancement of miRNA function have demonstrated great promise for counteracting cardiac diseases (22, 23). However, the off-target effects of miRNAs and antisense oligonucleotides that target miRNAs (i.e. the pleiotropic action of miRNA, affecting multiple genes in different tissues) may prevent the progression of miRNA regulation to therapeutic applications (24).

In the current study, we report that many XNAs bind PLN with similar strong affinity to what was found previously for SPIDRs (12). These molecules, which are tunable by length, would allow clinicians to match the reversal of SERCA inhibition to the severity of the disease. In addition, we found that most XNAs display similar functional effects to what was seen for ssDNA, specific miRNAs (both miR-1 and miR-21) have a greater effect on the SERCA-PLN complex activity than similar length random sequence RNA libraries. Furthermore, our results suggest that endogenous, non-coding miRNAs may play a more complex role in cardiac regulation than previously thought, targeting SERCA-PLN function via direct physical interactions.

**Results**

**RNA Sequences Bind Phospholamban with Low Nanomolar K_d Reversing SERCA Inhibition**—Previously, we found that ssDNA of varying lengths could relieve the inhibition of SERCA by PLN to different extents (12). We also determined that a 50-mer of RNA bound PLN with similar affinity and relieved inhibition of SERCA to a similar extent as ssDNA (12). In the current study, we determined the full extent of different lengths of RNA binding to PLN and its functional effects on the SERCA-PLN complex. Because non-coding RNAs are abundant in cardiomyocytes, we assayed whether RNA sequences would interact with PLN and reverse SERCA inhibition (Figs. 1 and 2). Using affinity capillary electrophoresis (ACE) and fluorescence polarization (FP), we found that random sequence RNA oligonucleotides (5–50-mers) have high affinity for PLN if longer than a 5-mer (Fig. 1). The 20-mer library, which is representative of the typical length of endogenous miRNAs, shows 9–19 nM binding affinity (Fig. 1). RNA demonstrated no measurable affinity for SERCA (K_d/4 M). Similarly, only weak affinity was observed for a group of cardiac proteins including actin, myosin, and troponin, suggesting that the observed RNA affinity is specific for PLN.

We performed a competitive assay to determine the contribution of the FAM (fluorescein) label to binding. As shown in Fig. 1B, unlabeled, random sequence, 20-mer RNA effectively competes with the FAM-labeled RNA library at the PLN binding site. The slope of the curve is 1.3 ± 0.07, suggesting that if anything, the addition of the FAM label diminishes the affinity of RNA for PLN.
RNAs of different lengths relieve PLN-mediated inhibition of SERCA. Coupled enzyme assays use a reconstituted lipid system containing SERCA and PLN and are commonly used to determine SERCA activity (25, 26). These assays determine the ATPase activity under near physiological conditions (26). SERCA affinity for calcium is decreased upon the addition of PLN (Fig. 2). The pK_{Ca} corresponds to the pCa at half-maximal activity (pCa at “normalized SERCA activity” = 0.5). The extent of PLN inhibition is quantified by a shift in the pK_{Ca} to lower values and can be visualized in Fig. 2 by the shift in the calcium curve to the right upon the addition of PLN. Upon the addition of RNA, the apparent calcium affinity of SERCA is augmented and the activity curve shifts back toward the left (higher pK_{Ca} values) (Fig. 2). The extent of relief of inhibition of SERCA is dependent on RNA length (Fig. 2). As RNA length increases, inhibition is gradually relieved, with the 50-mer re-establishing SERCA basal activity. This is similar to the incremental relief we have previously observed for ssDNA (12). The random sequence library that was used in these experiments confirms that the RNA-PLN interaction is sequence-independent. These results imply that cytosolic miRNAs are able to interact with the SERCA-PLN complex, providing a natural mechanism of regulation through direct, physical interaction with PLN, a novel phenomenon that has not been seen before with a non-transcription related protein.

miR-1 and miR-21 Bind to PLN Strongly and Relieve Inhibition of SERCA—To determine whether endogenous miRNA sequences are able to reverse the inhibition of PLN, we chose miR-1 and miR-21, which are 22 and 21 nucleotides in length (Fig. 3A). Both miR-1 and miR-21 are implicated in many disease states (13), and miR-1 is highly abundant in cardiomyocytes (13). Using ACE and FP, we found that both miR-1 and miR-21 bind PLN tightly, with low nM dissociation constants. Although displaying slightly higher dissociation constants (~40–60 nM) than the random sequence mixture (~10 nM), they are still able to bind with remarkably high affinity (Fig. 3, B–D). The difference in K_d may be due to the presence of secondary structure in both miR-1 and miR-21, which may incur a thermodynamic penalty associated with unfolding to bind PLN. The secondary structures, as predicted by mfold (University of New York at Albany), are shown in Fig. 3A. Nonetheless, both miR-1 and miR-21 effectively reverse PLN inhibition and fully restore the basal activity of SERCA more efficiently than the random mixture of 20-nucleotide RNA sequences (Fig. 3E).
Both miR-1 and miR-21 remove all PLN-mediated inhibition. The resulting $pK_{Ca}$ is not statistically different from SERCA alone (Fig. 3E). This suggests that miRNAs may have sequence elements that relieve inhibition more effectively than random sequence RNA.

Oligonucleotide Analogs (XNAs) Bind Phospholamban with Low Nanomolar Dissociation Constants—We analyzed several modified oligonucleotide analogs to determine the specific chemical moieties of ssDNA and RNA that are responsible for the high affinity for PLN (Fig. 4). These chemically modified oligonucleotides (XNAs) also display increased nuclease resistance that is essential for in vivo applications. The XNAs assayed include nuclease-resistant and chemical modifications of DNA such as L-DNA (the enantiomer of natural DNA), 2'-O-methyl RNA, phosphorothioate DNA (PT DNA, replacement of one of the non-bridging oxygens with a sulfur), 2'-O-methyl (2'-O-Me) RNA (the addition of an -OCH$_3$ group to carbon 2), L-DNA (the enantiomer of natural DNA), PNA (neutral peptide backbone with DNA bases), and morpholinos (neutral backbone).

We found that charged XNAs (i.e. PT DNA, L-DNA, and 2'-O-methyl RNA) bind PLN with an affinity similar to ssDNA or RNA ($K_d < 4$ nm). Neutral oligonucleotides (i.e. PNA and morpholinos) did not demonstrate any measurable affinity for PLN (Fig. 5), suggesting that affinity is driven by the negatively charged backbone. The cytoplasmic domain of PLN has four positively charged amino acids (Lys-3, Arg-9, Arg-13, and Arg-14) that we previously implicated in interactions with the negatively charged backbone of ssDNA (12). It should be noted that there are other well known examples of proteins, including Taq polymerase (27–29) and histones (30, 31), that interact with nucleic acids primarily through electrostatic and polar interactions at the phosphodiester backbone in a sequence-independent manner.

XNAs Relieve the Inhibition of SERCA by PLN to Different Extents—We also performed coupled enzyme assays using the same lipid-reconstituted system as in Fig. 2. For these experi-
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FIGURE 6. Oligonucleotide analogs (XNAs) relieve PLN-mediated inhibition of SERCA. A, normalized SERCA ATPase activity was measured at increasing Ca\(^{2+}\) concentrations using a coupled enzyme assay in DOPC:DOPE lipid vesicles. The black line is the activity curve for SERCA alone; the red line is SERCA + PLN, while the other colors represent the addition of different 20-mer, random sequence libraries of various oligonucleotide analogs to the SERCA + PLN mixture. A regression analysis (Equation 1) was used to estimate \(pK_{Ca}\) (i.e. \(pCa^{2+}\) at activity half-maximum) from each curve. Each data point is the average of \(n = 6\) replicates. Error bars were omitted for clarity. B, \(pK_{Ca}\) estimates from A are plotted for different oligonucleotide analogs (20-mer, random sequence). Error bars are the standard errors from the regression analyses used to estimate \(pK_{Ca}\) (Equation 1, \(n = 6\) replicates measured at 12 Ca\(^{2+}\) concentrations). \(p\) values were calculated using an unpaired \(t\) test (*, \(p < 0.05\), ***, \(p < 0.001\)).

Discussion

Our previous work demonstrated that random sequence ssDNA could be used in a tunable manner to alter SERCA function both in vitro and in vivo. However, ssDNA is not a viable therapeutic due to its short in vivo lifetime. Therefore, we turned to XNAs for two reasons: 1) with their nuclease-resistant properties, they have a much longer in vivo lifetime (32) and are potential therapeutic candidates if a suitable carrier can be developed, and 2) the properties of XNAs enabled us to understand the chemical requirements behind this high affinity interaction between random sequence ssDNA and PLN.

We confirmed that RNA \(>5\) nucleotides long tightly binds PLN (Fig. 1). Additionally, RNA demonstrates a similar length-dependent relief of inhibition (Fig. 2) to what was seen for ssDNA on SERCA activity (12). PLN-mediated inhibition of SERCA is removed in a stepwise, tunable manner with increasing lengths of RNA. Furthermore, we demonstrated that electrostatics are necessary for strong affinity for PLN (Figs. 5 and 6); all RNA molecules with a negatively charged backbone display high affinity for PLN. However, electrostatics are not the only important factor when considering relief of SERCA inhibition (Fig. 6). In fact, 2’-O-methyl RNA displays a similar affinity for PLN as ssDNA, but does not demonstrate a similar relief of inhibition. The addition of the methoxy group on the 2’-carbon may sterically hinder the structural conformation or eliminate a hydrogen-bonding site needed for relief of SERCA function.
The homeostatic balance of Ca\(^{2+}\) cycling in cardiac muscle cells is essential for proper function (1, 2, 33). SERCA and PLN are key proteins within this tightly regulated process (3) and have been identified as potential drug targets (34, 35). Improper regulation of SERCA function, either by PLN mutants or by changes in PLN phosphorylation level, leads to cardiomyopathies (36–45). To reverse adverse effects of heart failure, Hajjar and co-workers (6) pioneered the use of gene therapy to augment Ca\(^{2+}\) transport. Unfortunately, their recent clinical trials did not show any improvement with respect to patients treated with placebo. In a parallel approach, chemically modified oligonucleotide analogs have been used to target miRNAs for calcium-handling proteins. Recently, this approach was used by Größl et al. (46) to target PLN. They constructed an adeno-associated virus (AAV) vector (scAAV6-amiR155-PLBr) to express an anti-miRNA (amiR155-PLBr) that enhanced Ca\(^{2+}\) transport into the sarcoplasmic reticulum. Here, we propose to use XNAs to directly target the SERCA-PLN complex rather than the miRNA transcripts.

Although random sequence ssDNAs elicit the same effects as XNA in a tunable manner both in vitro and in cellular experiments (12), ssDNA is not a viable therapeutic agent due to its short in vivo lifetime. XNAs, on the other hand, display similar binding properties and activity toward the SERCA-PLN complex, with the advantage of being nuclease-resistant, i.e. possessing a longer in vivo lifetime. Therefore, XNAs, or related small molecules, could be developed into targeted therapeutics that directly interact with the SERCA-PLN complex to relieve inhibition in a tunable manner. This type of design flexibility using molecule length, not concentration, is unique and biologically important because it leads to potential customizable drug development. The length and properties of the molecule could be tuned to provide a graded response, allowing for varying degrees of relief of inhibition based on disease severity. This would be the first customizable therapeutic directly targeting SERCA function. Our studies will pave the way for developing alternative therapeutic approaches to treat cardiomyopathies focused on the SERCA-PLN complex.

An intriguing aspect of miRNA biology is the up- and down-regulation of specific miRNAs in response to cardiac stress (13, 47, 48). The presence of miRNAs in the cytoplasm under pathological conditions suggests that they may be able to interact with cardiac proteins directly, modifying their functions. We demonstrated, for the first time, the potential for miRNAs to physically interact with and change the function of non-transcriptionally related proteins. Specifically, we examined miR-1 and miR-21 as examples of miRNAs with the potential to interact directly with proteins in vivo. We chose these two miRNAs because they have important roles in cardiomyocyte biology. miR-1 is highly conserved throughout species, is the most abundant miRNA in human adult hearts (~24%), and is also lethal in homozygous knockdown mice (13, 15, 49). miR-21 is one of the most consistently up-regulated miRNAs in cardiac hypertrophy, and inhibition of miR-21 enhances hypertrophic growth (13, 47, 48). We demonstrated that both miR-1 and miR-21 bind PLN and relieve inhibition of SERCA (Fig. 3). Intriguingly, the specific sequences of miR-1 and miR-21 are more effective at relieving inhibition than a random sequence mixture of 20-mer RNA. Both miR-1 and miR-21 completely relieve the inhibition by PLN, fully restoring SERCA function (Fig. 3E). Considering the high affinity of the miRNA-PLN interactions and the abundance of miRNAs in cardiac cells, it would be surprising if these interactions did not take place in vivo. The increased effectiveness of naturally occurring miRNAs suggests that they may have evolved to contain sequence elements that are particularly effective at relieving PLN inhibition. Together these results support the existence of a previously unreported regulatory role for miRNAs where they are able to modulate function through direct protein interactions.

### Experimental Procedures

**Chemicals**—Unless otherwise noted, oligonucleotides (ssDNA, RNA, 2’-O-methyl RNA, and PT DNA), were purchased from Integrated DNA Technologies, Inc. PNA were purchased from PNA Bio, morpholinos were purchased from Gene Tools, LLC, and 1'-DNA was purchased from ChemGenes Corp. All ssDNA, RNA, PT DNA, and 2’-O-methyl sequences were synthesized as random sequence mixtures. All oligonucleotides were purchased in duplicate, one with a FAM label for the binding studies and a duplicate without the label for the activity assay studies. Phospholipids (1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE)) were purchased from Avanti Polar Lipids, and octa-ethylene glycol monododecyl ether (C\(_{12}\)E\(_{9}\)) was purchased from Anatrace. The following were purchased from Sigma: NADH (N6785), MgCl\(_2\) (M2670), KCl (P9333), phosphoenolpyruvate (P3637), ATP (D6500), lactate dehydrogenase (L3916), and pyruvate kinase (P-1506). MOPS (M92020) and glycerol were purchased from Research Products International. Imidazole (AC301872500) and sucrose (BP220212) were purchased from Fisher Scientific. EGTA was purchased from VWR Scientific, and the calcium ionophore A23187 was purchased from A. G. Scientific.

The binding buffer for ACE and FP was as follows: 20 mM MOPS, pH 7.0, 1 mM MgCl\(_2\), 5 mM KCl, 0.1% (w/v) C\(_{12}\)E\(_{9}\). The buffers used for the activity assays include the reconstitution buffer (25 mM imidazole, pH 7.0, 100 mM KCl, 5 mM MgCl\(_2\), 10% glycerol, 0.2% C\(_{12}\)E\(_{9}\)) and the assay buffer (100 mM MOPS, pH 7.0, 200 mM KCl, 5.0 mM MgCl\(_2\), 2.0 mM EGTA, 0.4 mM NADH, 1.0 mM phosphoenolpyruvate, 5.0 mM ATP, 0.00712 mg/ml calcium ionophore A23187, 10 IU/ml lactate dehydrogenase, and 10 IU/ml pyruvate kinase). Calcium solutions for the coupled enzyme assays were prepared according to Ref. 45. using CaCl\(_2\) to pH values of 5.0–8.0 in 20 mM MOPS, 0.3 M sucrose at pH 7.0.

**Protein Purification**—Phospholamban expression and purification were achieved using previously published methods (50). SERCA (SERCA1a isoform) was purified from the sarcoplasmic reticulum of rabbit skeletal muscle using affinity chromatography and previously reported methods (51).

**SERCA Activity Assays**—The reconstituted system containing either SERCA alone or SERCA with recombinant PLN was reconstituted in multimellar lipid vesicles using DOPC and DOPE at a 4:1 DOPC:DOPE molar ratio (26). XNA solutions were prepared using nuclease-free water. XNAs were added
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directly to the reconstituted vesicles containing SERCA and PLN and incubated for 25 min prior to the ATPase assay. The SERCA-PLN complex was reconstituted to a final molar ratio of 700:10:1 lipid:PLN:SERCA. The coupled enzyme assay measures the calcium dependence of the ATPase activity of SERCA using a previously established method (25). The enzymatic activity at 37 °C was monitored by following the decrease of NADH absorbance (A340) with a SpectraMax microplate reader (Molecular Devices) equipped with a 96-well plate. The initial rate of SERCA activity was measured as a function of calcium concentration and then fit to the Hill equation (Equation 1) using Origin 9.1

\[
V = \frac{V_{\text{max}}}{1 + 10^{nK_p\text{Ca} - p\text{Ca}}} \quad \text{(Eq. 1)}
\]

where \( V \) is the initial rate; \( V_{\text{max}} \) is the maximum rate; \( p\text{Ca} \) is the negative logarithm of calcium concentration; \( pK_p\text{Ca} \) is the \( p\text{Ca} \) value where \( V = V_{\text{max}}/2 \), and \( n \) is the Hill coefficient. Data sets were normalized to \( V_{\text{max}} \).

Affinity Capillary Electrophoresis—All experiments were performed on a Beckman Coulter commercial capillary electrophoresis system (P/ACE MDQ) with laser-induced fluorescence detection at \( \lambda_{\text{ex}} = 488 \text{ nm} \), \( \lambda_{\text{em}} = 520 \text{ nm} \). All sequences were labeled with FAM fluorophores for detection. FAM-labeled XNAs at a concentration of 10 nm were mixed with increasing concentrations of PLN and injected into a 40 cm × 50-μm fused silica capillary (Polymicro Technologies, Phoenix, AZ) using hydrodynamic injection (1 p.s.i. for 4 s). ACE separations were performed in reverse polarity mode at 30 kV for 9 min. Electropherograms were analyzed using Cuter 7.0 (52), where the peaks heights and area of the free XNA peak were used to determine the \( K_p \) according to Ref. 53,

\[
f_b = \frac{c}{1 + K_p} \times \frac{1}{[P]_i \cdot 0.5([D] + [P]_h + K_o - ([D] + [P]_h + K_o)^2 - 4[D][P])} \quad \text{(Eq. 2)}
\]

where \( f_b \) is the bound fraction; \( c \) is the maximum bound fraction; and \([P]_i\) and \([D]_i\) are the total concentrations of PLN and DNA, respectively. The denominator represents the free concentration of PLN.

Fluorescence Polarization—FP experiments were performed using a Synergy™ 2 microplate reader from BioTek Instruments, Inc. (Winooski, VT). All FP experiments with XNAs were performed using filter settings as follows: \( \lambda_{\text{ex}} = 485 \pm 20 \text{ nm} \), \( \lambda_{\text{em}} = 528 \pm 20 \text{ nm} \). Samples were made identically to the ACE samples. From these samples, 15-μl aliquots were loaded into a Corning 384-well plate (3540 microplate from Corning Inc., Corning, NY). Parallel and perpendicular intensities were measured with a sensitivity setting of 120, and polarization values were calculated from these values. Calibrations were performed using the G-factor, which was calculated in the Gen 5TM software (BioTek Instruments, Inc., Winooski, VT). Bound fractions of XNA were determined according to

\[
f_b = \frac{P - P_o}{P_m - P_o} \quad \text{(Eq. 3)}
\]

where \( f_b \) is the bound fraction; and \( P, P_m \) and \( P_o \) are the measured polarizations of a sample, complex, and free ssDNA, respectively. Polarization of the complex (\( P_m \)) is the plateau value at a saturating concentration of PLN. The \( K_d \) was then determined from fitting the data to Equation 2 using Origin 9.1. Overall fluorescence intensities of the samples were monitored, and the bound fraction was modified if the overall fluorescence intensity was biased, according to the published method (53).

**Competition Assay**—Solutions of 100 nm FAM-labeled, random sequence, 20-mer RNA + 100 nm PLN were prepared with 0–500 nm unlabeled, random sequence, 20-mer RNA. Fluorescence polarization of each solution was measured to plot the fraction of labeled RNA dissociated from PLN versus mole fraction of unlabeled RNA (i.e. \( [\text{RNA}]_{\text{unlabeled}}/[\text{RNA}]_{\text{total}} \)). Fluorescence polarization of 100 nm FAM-labeled, 20-mer RNA alone was measured as the “free labeled RNA” control.

**Author Contributions**—K. J. S., J. Y., G. V., and M. T. B. conceived and designed the work. K. J. S. and J. Y. conducted the experiments. K. J. S., J. Y., G. V., and M. T. B. analyzed the results. K. J. S. and M. T. B. wrote the paper with insight from G. V.

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