# Phospholamban Thiols Play a Central Role in Activation of the Cardiac Muscle Sarcoplasmic Reticulum Calcium Pump by Nitroxyl†

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ABSTRACT: Nitroxyl (HNO) donated by Angeli's salt activates uptake of $Ca^{2+}$ by the cardiac SR $Ca^{2+}$ pump (SERCA2a). To determine whether HNO achieves this by a direct interaction with SERCA2a or its regulatory protein, phospholamban (PLN), we measured its effects on SERCA2a activation (as reflected in dephosphorylation) using insect cell microsomes expressing SERCA2a with or without PLN (wild-type and Cys → Ala mutant). The results show that activation of SERCA2a dephosphorylation by HNO is PLN-dependent and that PLN thiols are targets for HNO. We conclude that HNO produces a disulfide bond that alters the conformation of PLN, relieving inhibition of the $Ca^{2+}$ pump.

Stimulation of the cardiac SR $Ca^{2+}$ pump (SERCA2a)[1] in isolated SR vesicles by cAMP/protein kinase A-dependent phosphorylation of phospholamban (PLN) was originally demonstrated by Tada et al. (*1*). Subsequent studies showed that phosphorylation of PLN at $Ser^{16}$ increases the apparent $Ca^{2+}$ affinity of SERCA2a (*2*), the rates of phosphorylation and dephosphorylation (*2, 3*), and the $V_{max}$ of $Ca^{2+}$ transport (*3, 4*). Phosphorylation of $Ser^{16}$ induces a change in the conformation of PLN (*5, 6*), which relieves its inhibition and activates the pump. Recent work with expressed $Ca^{2+}$ pump proteins suggests that activation of the cardiac SR $Ca^{2+}$ pump following the relief of PLN inhibition involves changes in the kinetic behavior of the $Ca^{2+}$-ATPase consistent with SERCA2a oligomerization (*7*). These changes include stabilization of the ADP-sensitive phosphoenzyme, E1P, and allosteric activation of dephosphorylation by ATP, resulting in an increased rate of turnover of the ADP-insensitive phosphoenzyme, E2P.

We have recently shown that nitroxyl (HNO) donated by Angeli's salt (AS, $Na_2N_2O_3$) activates the SR $Ca^{2+}$ pump in isolated murine cardiac myocytes (*8*). This effect is independent of $\beta$-adrenergic activation (*9*) and results from a direct effect of HNO on the SR $Ca^{2+}$ pump as evidenced by stimulation of $Ca^{2+}$ uptake by HNO in isolated murine heart SR vesicles (*8*). We hypothesize that HNO stimulates $Ca^{2+}$ uptake by covalently modifying critical thiol residues in the SERCA2a pump and/or its regulatory protein, PLN, altering the conformation of these proteins, and relieving the inhibition of the pump. Chemical modification of protein thiols by HNO can proceed by two pathways leading to either (a) the formation of a sulfinamide [$RS(O)NH_2$] when one thiol group is involved or (b) the formation of a disulfide (RSSR) plus hydroxylamine ($H_2NOH$) when two thiols are in the proximity of each other (*10, 11*). The fact that HNO-induced activation of RyR2 receptors reconstituted in lipid planar bilayers can be reversed by DTT (*8*) suggests that disulfide bond formation may be important in activating $Ca^{2+}$ release.

In this study, we investigated the mechanism of activation of the SR $Ca^{2+}$ pump by HNO using SERCA2a expressed in the absence or presence of PLN in High Five (HF) insect cell microsomes. To study $Ca^{2+}$ pump activation by HNO, we measured the kinetics of dephosphorylation of SERCA2a, which is accelerated by HNO. To test whether thiol residues in PLN are critical for activation, we carried out these experiments in microsomes expressing SERCA2a and null-Cys PLN [PLN(−C)] in which the three transmembrane (TM) domain cysteine residues were replaced with alanine. Immunoblotting with an anti-PLN antibody was used to test whether HNO-induced PLN oligomer formation contributes to the relief of inhibition of SERCA2a by PLN. The results show that PLN is necessary for activation of SERCA2a by HNO and that cysteine residues in the TM domain of PLN play a critical role in PLN-dependent HNO activation of the $Ca^{2+}$ pump.

We previously showed (*7*) that expression of SERCA2a without PLN in HF insect cells activates turnover of E2P in the $Ca^{2+}$-ATPase reaction mechanism. To determine whether HNO activates the $Ca^{2+}$ pump by a similar mechanism, we phosphorylated ER microsomes expressing SERCA2a and WT PLN with ATP and then chased with EGTA prior to

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[1] Abbreviations: SR, sarcoplasmic reticulum; SERCA2a, cardiac muscle isoform of the sarco(endo)plasmic reticulum $Ca^{2+}$-transporting adenosine triphosphate; PLN, phospholamban; EP, phosphoenzyme; E1P, ADP-sensitive phosphoenzyme; E2P, ADP-insensitive phosphoenzyme; EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-*N*′,*N*′,*N*′,*N*′-tetraacetic acid; TM, transmembrane; WT, wild-type; HF, High Five; AS, Angeli's salt; HNO, nitroxyl; DTT, dithiothreitol; NEM, *N*-ethylmaleimide.

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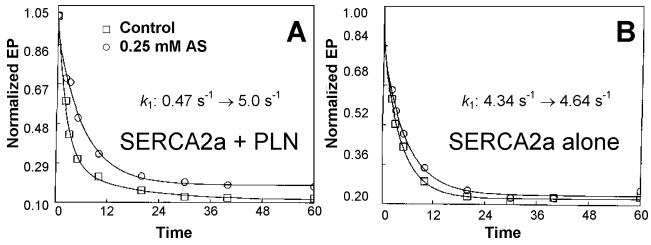


FIGURE 1: Kinetic effects of HNO on dephosphorylation of SERCA2a in HF insect cell microsomes expressing SERCA2a and WT PLN (A) or SERCA2a alone (B). Microsomes expressing canine SERCA2a with or without WT PLN were isolated from HF insect cells and incubated with 0 (□) or 0.25 mM AS (○) for 25 min at 24 °C. The microsomes were phosphorylated at 0 °C with 0.1 mM [$\gamma$-$^{32}$P]ATP for 10 s and then dephosphorylated with 5 mM EGTA before being quenched with acid (see the Supporting Information). The amplitudes and rate constants for phosphoenzyme decay presented in Table 1 were evaluated by modeling the data using nonlinear regression curve fitting (see the Supporting Information).

quenching with acid (Figure 1A). EGTA prevents rephosphorylation of the enzyme by chelating the free $Ca^{2+}$, allowing the spontaneous turnover of E2P to be assessed. In the absence of AS, the time course of dephosphorylation exhibited two slow decay phases (0.4 and 0.1 $s^{-1}$) and a stable residual component (Table 1). Incubation with AS at a concentration (0.25 mM) that activates $Ca^{2+}$ uptake in cardiac SR vesicles (*8*) elicited a fast phase of dephosphorylation with a rate constant (4.9 $s^{-1}$) 10-fold higher than that observed in the absence of AS. Microsomes expressing SERCA2a alone exhibited a biphasic EP decay pattern with a fast phase (4.7 $s^{-1}$) that was not significantly different from the fast phase in AS-treated microsomes expressing SERCA2a and PLN (Table 1). When SERCA2a-expressing microsomes were incubated with 0.25 mM AS, the kinetics of the fast phase of dephosphorylation was unchanged (4.5 $s^{-1}$) compared to the control (Figure 1B). This implies that the SR $Ca^{2+}$ pump in microsomes expressing SERCA2a alone is in an activated state prior to exposure to AS and that subsequent addition of AS does not lead to a further activation of the pump. Moreover, it demonstrates that activation by HNO is PLN-dependent since the addition of AS to microsomes expressing SERCA2a alone had no effect on the kinetics of dephosphorylation (Table 1).

The effects of glutathione in cardiac myocytes and DTT on the reconstituted RyR2 receptor activated by HNO (*8*) suggest that critical thiols in SR proteins play an important role in the HNO-mediated effects. PLN contains three cysteine residues (36, 41, and 46), all in the transmembrane domain, that are potential targets for HNO. To test this possibility, we coexpressed SERCA2a with mutant PLN in which all three cysteines were replaced with alanine [PLN(−C)]. In contrast to microsomes expressing SERCA2a with WT PLN, dephosphorylation of SERCA2a expressed with PLN(−C) exhibited a slow monoexponential decay phase (0.2 $s^{-1}$) that was not accelerated (0.2 $s^{-1}$) by the addition of 0.25 mM AS (Table 1 and the Supporting Information). The SERCA2a pump in these microsomes was still under the regulatory control of the mutant PLN since incubation with anti-PLN antibody 2D12 (*12*) led to activation of $Ca^{2+}$ uptake (not shown).

Our results showing that activation of SERCA2a is PLN-dependent and that SERCA2a coexpressed with PLN lacking cysteines cannot be activated by HNO point to thiol reactivity with HNO and potential disulfide bond formation. To test this possibility, we compared the kinetics of dephosphorylation in SERCA2a with WT PLN microsomes treated with 0.25 mM AS alone to the behavior in microsomes treated with 0.25 mM AS followed by 5 mM DTT. As seen in Table 1, exposure to DTT largely restored the kinetic parameters of dephosphorylation to those observed under the baseline condition. This suggests that disulfide bond formation in PLN plays a major role in the activation of SERCA2a and that breaking these bonds restores the inhibition by PLN.

Since the inhibition of SERCA2a by PLN involves the monomeric form of this regulatory protein, interchain disulfide bonds formed between PLN monomers could also activate the $Ca^{2+}$ pump by sequestering PLN in a noninhibitory oligomeric complex. We tested this by incubating microsomes expressing PLN with varying concentrations of AS (0−10 mM) and assessing the appearance of cross-linked PLN monomers by immunoblotting with an anti-PLN antibody (Figure 2A). As expected (*13*), untreated PLN migrates as both a monomer and a homopentamer, and boiling the samples results in dissociation of the homopentamer. With added AS, a new very faint band is observed that, on the basis of comparison with standard molecular weight markers, is assigned to the PLN dimer. No intermolecular cross-linking was observed between PLN and SERCA2a (not shown). PLN dimer intensity increased with an increase in the concentration of added AS (Figure 2A,B) but remained a minor component without a detectable decrease in the monomer or pentamer bands. Boiling the sample, which converts the pentamer to the monomer, increased the dimer fraction observed with ≥5 mM AS, indicating that some of the initially formed dimer takes part in pentamer formation. These results demonstrate that only a small fraction of PLN molecules are sequestered in the dimer following exposure to 0.25 mM AS and that the primary pathway for activation of the cardiac SR $Ca^{2+}$ pump by HNO likely involves intramolecular disulfide formation.

Additional experiments (see the Supporting Information for details) were performed that confirm HNO targets PLN cysteine residues to form disulfides. (a) PLN dimer formation is eliminated by preblocking the cysteine residues with NEM and is suppressed by the addition of glutathione, which competitively traps HNO in a concentration-dependent manner. (b) No dimer formation is seen in experiments with microsomes containing PLN(−C). (c) PLN-expressing microsomes incubated with 0.25 mM AS and then exposed to increasing concentrations of DTT (0−50 mM) exhibited a concentration-dependent reduction in PLN dimer band intensity (Figure 2C). (d) DTT is unreactive with a model sulfinamide (*tert*-butanesulfinamide), suggesting that disulfides rather than sulfinamides are formed by the reaction of HNO with PLN cysteines. (e) PLN dimer formation is also observed under anaerobic conditions, indicating that HNO, rather than an HNO−oxygen adduct (*14*), is the reactive species.

These studies involving mutant PLN and successive exposure to AS and DTT strongly support the hypothesis that thiols in PLN are targets for HNO and that disulfide bond formation is important in relieving its inhibition of SERCA2a. Moreover, the covalent modification of PLN thiols to sulfinamides by HNO is excluded by the lack of reactivity of DTT with sulfinamides. Current models of PLN

Table 1: Effect of PLN (WT), Mutant (Cys → Ala) PLN, and DTT on the Kinetics of SERCA2a Dephosphorylation Activated by HNO[a]

| preparation | treatment | $A_1$ | $k_1$ ($s^{-1}$) | $A_2$ | $k_2$ ($s^{-1}$) | $R$ |
|---|---|---|---|---|---|---|
| SER with WT PLN | none | $0.7 \pm 0.2$ | $0.4 \pm 0.3$ | $0.2 \pm 0.1$ | $0.1 \pm 0.1$ | $0.1 \pm 0.1$ |
| SER with WT PLN | 0.25 mM AS | $0.2 \pm 0.1^b$ | $4.9 \pm 0.3^b$ | $0.6 \pm 0.1^b$ | $0.2 \pm 0.1^b$ | $0.2 \pm 0.1^b$ |
| SER with WT PLN | AS, then DTT | $0.6 \pm 0.1$ | $0.7 \pm 0.3$ | $0.3 \pm 0.2$ | $0.1 \pm 0.1$ | $0.1 \pm 0.1$ |
| SER alone | none | $0.2 \pm 0.1^b$ | $4.7 \pm 0.3^b$ | $0.6 \pm 0.1^b$ | $0.2 \pm 0.1^b$ | $0.2 \pm 0.1^b$ |
| SER alone | 0.25 mM AS | $0.2 \pm 0.1^b$ | $4.5 \pm 0.2^b$ | $0.6 \pm 0.1^b$ | $0.2 \pm 0.1^b$ | $0.2 \pm 0.1^b$ |
| SER with PLN(−C) | none | $0.8 \pm 0.1$ | $0.2 \pm 0.1$ | | | $0.2 \pm 0.1$ |
| SER with PLN(−C) | 0.25 mM AS | $0.8 \pm 0.1$ | $0.2 \pm 0.1$ | | | $0.2 \pm 0.1$ |

[a] Results are reported as mean ± the standard deviation, where $n = 3$. Abbreviations: SER, SERCA2a; PLN, WT phospholamban; PLN(−C), phospholamban lacking cysteines (replaced with alanines). Kinetic parameters of dephosphorylation were modeled (MLAB) using mono- and biexponential decay functions including a residual component ($R$) (see the Supporting Information). EP decay was normalized prior to fitting so that $A_1 + A_2 + R = 1$. [b] Significantly different from the corresponding parameter in row 1 (SER with WT PLN, no treatment).

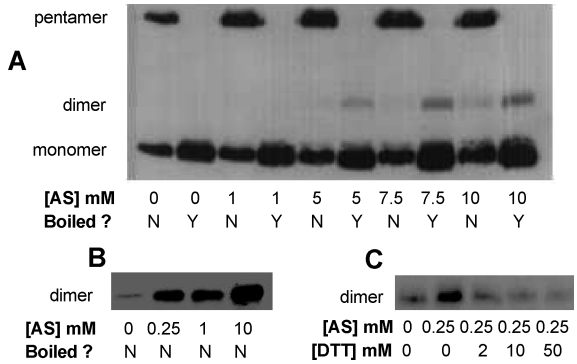


FIGURE 2: (A) Effect of AS and HNO on PLN dimer formation in HF insect cell microsomes with a short (30 s) exposure time. (B) Visualization of the PLN dimer with a prolonged (30 min) exposure time. (C) Effect of DTT on PLN dimer formation, also visualized with a prolonged (30 min) exposure time (see the Supporting Information).

transmembrane helix structure (*15*) suggest that the cysteine pairs (36 and 41, and 41 and 46) are not positioned properly to facilitate the formation of intramolecular PLN disulfide bonds by HNO. Wouters et al. (*16*), however, have recently reported that α-helical regions in certain proteins containing the sequence CXXXXC are able to form "forbidden" disulfides when an oxidizing agent is present, raising the possibility of an intramolecular disulfide bond in PLN. This hypothesis is strengthened by the fact that the reducing agent DTT relieved the activation by HNO, restoring the kinetics of the dephosphorylation reaction to its pre-HNO state (Table 1). Formation of an intramolecular bond could distort the conformation of PLN, thus perturbing its interaction with SERCA2a and relieving the inhibition. Subsequent reduction of the disulfide bond by DTT allows PLN to return to its pre-HNO conformation, re-establishing its contact with SERCA2a and restoring the inhibition. The physical change in PLN induced by HNO occurs in the TM domain, distinguishing it from the conformational effects of cAMP/PKA affecting the cytoplasmic domain. While these mechanisms are inherently different, they both lead to activation of E2P hydrolysis, which will increase the catalytic efficiency of the $Ca^{2+}$ pump by reducing rate limitation (*7*). Although this behavior is consistent with the chemistry of HNO and reactive thiols in proteins (*10, 16*), additional ongoing experiments are needed to establish that disulfide bonds in PLN have an essential role in the HNO-mediated effects both in vitro and in vivo and to determine which of the cysteine pairs in PLN (36 and 41, or 41 and 46) is crucial for activation.

## SUPPORTING INFORMATION AVAILABLE

General experimental procedures, data showing the effects of HNO on the kinetics of dephosphorylation of SERCA2a coexpressed with PLN(−C) (Figure S1), and data demonstrating that PLN dimer formation is eliminated by preblocking with NEM (Figure S2), suppressed by the addition of glutathione (Figure S3), not observed with PLN(−C) (Figure S4), and also observed under anaerobic conditions (Figure S5). This material is available free of charge via the Internet at http://pubs.acs.org.

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