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Activating and Deactivating Roles of Lipid Bilayers on the Ca^{2+}-ATPase/Phospholamban Complex

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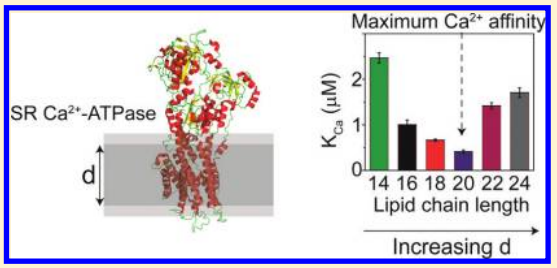
ABSTRACT: The physicochemical properties of the lipid bilayer shape the structure and topology of membrane proteins and regulate their biological function. Here, we investigated the functional effects of various lipid bilayer compositions on the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA) in the presence and absence of its endogenous regulator, phospholamban (PLN). In the cardiac muscle, SERCA hydrolyzes one ATP molecule to translocate two Ca^{2+} ions into the SR membrane per enzymatic cycle. Unphosphorylated PLN reduces SERCA's affinity for Ca^{2+} and affects the enzymatic turnover. We varied bilayer thickness, headgroup, and fluidity and found that both the maximal velocity (V_{max}) of the enzyme and its apparent affinity for Ca^{2+} (K_{Ca}) are strongly affected. Our results show that (a) SERCA's V_{max} has a biphasic dependence on bilayer thickness, reaching maximum activity with 22-carbon lipid chain length, (b) phosphatidylethanolamine (PE) and phosphatidylserine (PS) increase Ca^{2+} affinity, and (c) monounsaturated lipids afford higher SERCA V_{max} and Ca^{2+} affinity than diunsaturated lipids. The presence of PLN removes the activating effect of PE and shifts SERCA's activity profile, with a maximal activity reached in bilayers with 20-carbon lipid chain length. Our results in synthetic lipid systems compare well with those carried out in native SR lipids. Importantly, we found that specific membrane compositions closely reproduce PLN effects (V_{max} and K_{Ca}) found in living cells, reconciling an ongoing controversy regarding the regulatory role of PLN on SERCA function. Taken with the physiological changes occurring in the SR membrane composition, these studies underscore a possible allosteric role of the lipid bilayers on the SERCA/PLN complex.



Sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA) is a multidomain membrane-spanning enzyme that regulates muscle relaxation by Ca^{2+} translocation from the cytosol into the SR lumen.[1,2] SERCA is a P-type ATPase and transports two Ca^{2+} ions per hydrolyzed ATP.[3] Phospholamban (PLN) is the main endogenous inhibitor of SERCA activity in cardiomyocytes, and its inhibition is relieved upon phosphorylation at Ser 16 or Thr 17 of PLN.[4−7] Since SERCA and PLN are the main regulators of the cardiac output, they are targets for treatment of cardiac disease.[8,9]

SERCA function has been measured in several different systems including mammalian cell lines,[10] insect cell microsomes,[11] and reconstituted lipid vesicles.[12] In a reconstituted system, the bilayer lipid composition can be controlled, making it ideal for studying the effects of lipids on SERCA function. The effects of bilayer thickness, headgroup, and membrane fluidity have all been previously investigated. It was found that SERCA activity directly depends on bilayer thickness, with a maximum activity found for a lipid chain length of 18 carbons and a gradual decrease in activity for either thicker or thinner bilayers.[13,14] Later data from Cornea and Thomas support these results and, more importantly, showed that the effect of bilayer thickness is correlated to the degree of SERCA oligomerization.[15] The biochemical results were supported by molecular dynamics simulations, which show that SERCA can adapt to the thickness of the bilayer through small conformational changes but that this adaptation is not efficient in thin (14-carbon) bilayers.[16] Moreover, Squier and co-workers showed that the zwitterionic phosphatidylethanolamine (PE), which represents 16% and 27% of the skeletal and cardiac SR membrane lipid, respectively,[17] increases SERCA's maximal activity (V_{max}).[18] A similar activating effect was observed for the anionic lipids phosphatidylserine (PS) and phosphatidylinositol (PI) phosphate, which are present at lower concentrations in the SR membrane.[19] In addition, SERCA requires fluid, liquid crystalline bilayers to function properly (i.e., more active in 18:1 phosphocholine (PC) than 18:0 PC).[20] All of these SERCA activity studies based their conclusions on the maximum ATPase activity or the activity at a single Ca^{2+} concentration. Also, the functional effects of lipids on the SERCA/PLN complex have not been investigated.

Here, we analyzed the effects of bilayer thickness, headgroup composition, and fluidity for SERCA and the SERCA/PLN complex in reconstituted lipid vesicles, monitoring the ATPase activity using enzyme-coupled assays. We show how the apparent Ca^{2+} affinity (K_{Ca}) and V_{max} are strongly dependent on the nature of the lipid bilayer. Importantly, our data reveal how the lipid bilayer composition influences the regulation of SERCA by PLN. Our conclusions on model membranes are supported by

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activity measurements in lipid membranes extracted from rabbit skeletal muscle.

EXPERIMENTAL PROCEDURES

All synthetic lipids (Table 1) were purchased form Avanti Polar Lipids (Alabaster, AL). Skeletal SR lipids were extracted using an acidified solution of 2:1 chloroform:methanol.[17]

Table 1. Synthetic Lipids Used for Reconstitutions

lipid	chemical name
14:1$^{\Delta 9\text{-Cis}}$ PC	1,2-di(9*Z*-tetradecenoyl)-*sn*-glycero-3-phosphocholine
16:1$^{\Delta 9\text{-Cis}}$ PC	1,2-di(9*Z*-hexadecenoyl)-*sn*-glycero-3-phosphocholine
18:1$^{\Delta 9\text{-Cis}}$ PC (DOPC)	1,2-di(9*Z*-octadecenoyl)-*sn*-glycero-3-phosphocholine
18:1$^{\Delta 9\text{-Trans}}$ PC	1,2-di(9*E*-octadecenoyl)-*sn*-glycero-3-phosphocholine
18:2$^{\Delta 9,\Delta 12\text{-Cis}}$ PC	1,2-di(9*Z*,12*Z*-octadecadienoyl)-*sn*-glycero-3-phosphocholine
20:1$^{\Delta 11\text{-Cis}}$ PC	1,2-di(11*Z*-eicosenoyl)-*sn*-glycero-3-phosphocholine
22:1$^{\Delta 13\text{-Cis}}$ PC	1,2-di(13*Z*-docosenoyl)-*sn*-glycero-3-phosphocholine
24:1$^{\Delta 15\text{-Cis}}$ PC	1,2-di(15*Z*-tetracosenoyl)-*sn*-glycero-3-phosphocholine
18:1$^{\Delta 9\text{-Cis}}$ PE (DOPE)	1,2-di(9*Z*-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine
18:1$^{\Delta 9\text{-Cis}}$ PS (DOPS)	1,2-di(9*Z*-octadecenoyl)-*sn*-glycero-3-phospho-L-serine

SERCA1a was purified from rabbit skeletal muscle using a reactive red affinity column[21] and was reconstituted for functional assays according to a well-established protocol.[12,22−28] Briefly, the lipids solubilized in chloroform were dried under N_2(g) for several hours, and resuspended in a buffer containing 20 mM imidazole, 0.1 M KCl, 5 mM $MgCl_2$, 10% (v/v) glycerol, and octaethylene glycol monododecyl ether ($C_{12}E_8$) at 4:1 (w/w) $C_{12}E_8$:lipid ratio. SERCA (~1 mg/mL in 0.1% w/v $C_{12}E_8$) was added to the suspension to a final lipid:SERCA (mol/mol) ratio of 700:1. To remove the detergent, the samples were incubated with Biobeads SM2 (75:1 (w/w) Biobeads:$C_{12}E_8$) for 3 h at 30 °C (for experiments where the bilayer thickness or lipid chain saturation was varied) or 20 °C (for all other experiments). A temperature of 30 °C was chosen to ensure that lipids with relatively high gel−liquid crystal transition temperature (T_m) (i.e., 24:1 PC) were in the liquid crystalline phase during the incubation period. A coupled enzyme assay was used to monitor SERCA activity (hydrolysis of ATP) at 37 °C as a function of calcium concentration.[12] The rate of enzyme activity was measured as a decrease of NADH absorption at 340 nm using a Spectramax plate reader (Molecular Devices). Data were fit using the Hill equation

$$V = V_{max}\frac{[Ca^{2+}]^n}{[Ca^{2+}]^n + K_{Ca}{}^n}$$

to extract the maximum activity (V_{max}), Hill coefficient (n), and calcium concentration needed to achieve half maximal activity (K_{Ca}).

To monitor the inhibition of SERCA by PLN, we chose a monomeric mutant of PLN (AFA-PLN^{N27A}) that induces a larger shift in SERCA's apparent Ca^{2+} affinity than wild-type PLN (PLN^{wt}). Recombinant AFA-PLN^{N27A} and PLN^{wt} were expressed and purified as described previously.[29] The co-reconstitution of SERCA and PLN was identical to the free SERCA samples with the exception that PLN (AFA-PLN^{N27A} or PLN^{wt}) was dissolved in a 4:1 (v/v) mixture of trifluoroethanol/cholorform and dried together with the lipids at a molar ratio of 10:1 or 5:1 PLN:SERCA.

RESULTS

Effects of Lipid Chain Structure on SERCA Activity. The lipid chain structure is the main determinant of the fluidity as well as the thickness of the lipid bilayer.[30] To evaluate the effects of bilayer thickness on SERCA activity, we conducted ATPase assays in *cis*-mono-unsaturated PC bilayers of varied lipid chain length. PC lipids were utilized since they constitute the major component of the SR membrane.[17] In our experience, SERCA V_{max} varies between different rabbit preparations (2.9 ± 1.4 IU, average ± standard deviation for four preparations in 4:1 (w/w) 18:1$^{\Delta 9\text{-Cis}}$ PC (DOPC)/18:1$^{\Delta 9\text{-Cis}}$ PE (DOPE) bilayers), while K_{Ca} is highly reproducible (0.47 ± 0.01 μM). Thus, we express SERCA V_{max} as a relative value to our standard bilayers (DOPC or 4:1 (w/w) DOPC/DOPE), while K_{Ca} is reported as raw values. In Figure 1A,B, we show

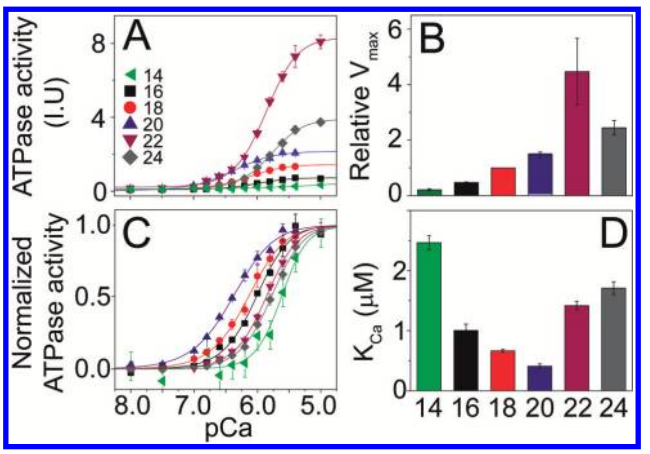

Figure 1. SERCA activity in bilayers of varying thickness. (A, C) Representative curves of raw and normalized ATPase activity as a function of calcium concentration in 14:1$^{\Delta 9\text{-Cis}}$, 16:1$^{\Delta 9\text{-Cis}}$, DOPC, 20:1$^{\Delta 11\text{-Cis}}$, 22:1$^{\Delta 13\text{-Cis}}$, and 24:1$^{\Delta 15\text{-Cis}}$ PC bilayers. Each point represents the average and standard error from three measurements. (B) V_{max} of SERCA relative to DOPC ($V_{max}/V_{max,DOPC}$) as a function of bilayer chain length. Error bars represent the standard error from at least three separate reconstitutions. (D) K_{Ca} in bilayers of varying thickness. Each bar was determined from the average of ≥3 reconstitutions, and error bars show the standard error of the mean.

that SERCA activity is highest in bilayers with a chain length of 22 carbons (22:1 bilayers) with a V_{max} value ~40-fold higher than in 14:1 bilayers, which had the lowest V_{max}. Previous studies, however, found that 18:1 bilayers show the highest SERCA activity.[13,15] The discrepancy might be due to the higher Ca^{2+} concentration used in the previous studies (10-fold excess relative to this study), which relied on measuring SERCA activity at only one Ca^{2+} concentration. Since our activity assays were measured for several Ca^{2+} concentrations (complete Ca^{2+} dependence curves), the fits to the Hill equation provide an accurate estimate of V_{max}. From the fitting of the activity curves, we also measured the SERCA K_{Ca} for each lipid length. Figure 1C,D shows that SERCA affinity for Ca^{2+} is biphasic, with the minimum K_{Ca} (highest apparent Ca^{2+} affinity)

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in 20:1 bilayers. Taken together, these data indicate that SERCA has the highest V_{max} in relatively thick bilayers (22:1), while bilayers with a thickness similar to the native SR membrane (average chain length of 18.1 carbons in cardiac SR and 17.8 carbons in skeletal SR)[17] give the highest affinity for Ca^{2+}. Therefore, the native SR lipid composition may be tuned to maximize the apparent Ca^{2+} affinity of SERCA.

Bilayer fluidity is another important physical property of biological membranes and SERCA requires a fluid bilayer to function.[20] To test the dependence of SERCA on bilayer fluidity, we reconstituted SERCA into $18{:}1^{\Delta 9\text{-Trans}}$ PC (similar bilayer thickness but less fluid than DOPC as determined by fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH))[14,31] and $18{:}2^{\Delta 9,\Delta 12\text{-Cis}}$ PC (similar bilayer thickness but more fluid than DOPC).[14,31] In $18{:}1^{\Delta 9\text{-Trans}}$ PC, SERCA V_{max} is slightly higher than in DOPC, while in $18{:}2^{\Delta 9,\Delta 12\text{-Cis}}$ PC V_{max} is significantly lower (Figure 2A). However, in both

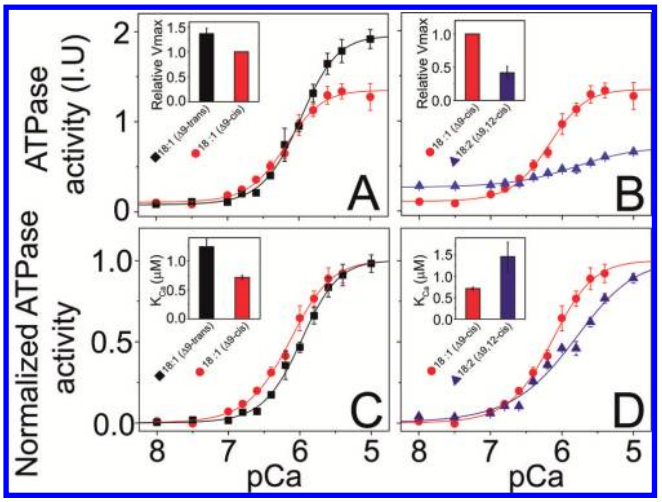

Figure 2. SERCA activity in C18 bilayers with different fluidity. (A, C) Raw and normalized ATPase activity in PC bilayers with differences in geometry around the unsaturated bond ($18{:}1^{\Delta 9\text{-trans}}$ PC vs DOPC ($18{:}1^{\Delta 9\text{-cis}}$ PC)) but identical length of the aliphatic chains. (B, D) ATPase activity in PC bilayers with differences in chain saturation (DOPC vs $18{:}2^{\Delta 9,\ \Delta 12\text{-cis}}$ PC) but identical length of the aliphatic chains. Error bars correspond to the standard error of three measurements. Averaged K_{Ca} and relative V_{max} are shown as insets, with the error bars representing the standard error of three or more separate reconstitutions.

$18{:}2^{\Delta 9,\Delta 12\text{-Cis}}$ PC and $18{:}1^{\Delta 9\text{-Trans}}$ PC bilayers K_{Ca} is significantly higher than in DOPC (Figure 2C,D). In the cardiac SR, the average number of unsaturated (*cis*) bonds per lipid chain is 1.6,[17] which is intermediate between that of DOPC and $18{:}2^{\Delta 9,\Delta 12\text{-Cis}}$ PC. Thus, both the thickness and fluidity of the SR bilayer are probably optimized for K_{Ca} rather than V_{max}.

An inherent shortcoming of our studies is that bilayer thickness and fluidity cannot be varied independently (i.e., $18{:}1^{\Delta 9\text{-Trans}}$ PC bilayers are thicker than DOPC bilayers). Therefore, it is important to emphasize that a change in SERCA activity cannot be explained by one single characteristic of the lipid bilayer. Nevertheless, the difference in bilayer thickness between DOPC and $18{:}1^{\Delta 9\text{-Trans}}$ PC is only ~1 Å[14] compared to the difference between 20:1 PC and 18:1 PC (~3 Å).[14] Thus, the difference in SERCA V_{max} and K_{Ca} between $18{:}1^{\Delta 9\text{-Trans}}$ PC and DOPC can be mainly attributed to the difference in fluidity between the two bilayers.

Effects of Phosphoethanolamine (PE) on the Activity of the SERCA/PLN Complex. Since the two major components of the native SR membrane are PC and PE lipids,[17] 4:1 (w/w) DOPC/DOPE lipid bilayers preparations have been used to study SERCA activity.[12,32] To determine the effects of PE on SERCA function, we carried out functional assays at different molar ratios of DOPC:DOPE. Since pure PE membranes form hexagonal phases and inhibit SERCA activity,[33] we limited the PE content to 60% (w/w) of the total lipid bilayer composition to retain the liquid crystalline phase.

When SERCA was reconstituted in the absence of PLN, we found that increased PE content steadily increases V_{max} at PE ratios greater than 20% (w/w), with no significant effects observed at PE content <20% (w/w) (Figure 3A,B). The latter is in agreement with previous studies.[18,33] Also, we found that the DOPC:DOPE bilayers have a significantly lower K_{Ca} compared to pure DOPC bilayers (Figure 3D,E). Thus, PE has an activating role on SERCA function, increasing both the apparent Ca^{2+} affinity and V_{max}.

PE is a curvature-forming lipid,[34] which can affect membrane protein function by inducing curvature and changing the lateral pressure of the membrane.[35,36] To test if this change in bilayer property could explain the effects of PE on SERCA function, we measured SERCA activity in the presence of phosphoserine (PS) lipids, which make up ~10% of the total SR membrane lipid content[17] and does not induce membrane curvature. In this case, we found that the 4:1 (w/w) PC:PS bilayers decreased SERCA V_{max} relative to both PC and 4:1 (w/w) PC:PE bilayers (Figure 3C). However, K_{Ca} values were slightly lower than those obtained in PC:PE (Figure 3F). The effects on K_{Ca} and V_{max} are present also at a physiological 9:1 (w/w) PC:PS ratio. These data show that PS increases the apparent Ca^{2+} affinity of SERCA and reveal a possible activating role of PS similar to PE lipids. This also suggests that the activation of SERCA by PE is not due to increased bilayer curvature.

In situ and *in vitro* experiments have shown that SERCA's apparent Ca^{2+} affinity is lowered in the presence of PLN.[11,12,23] To determine the effects of bilayer composition on SERCA inhibition by PLN, we co-reconstituted a monomeric, super-inhibitory mutant of PLN (AFA-PLN^{N27A})[12,37] with SERCA into lipid vesicles composed of DOPC or DOPC/DOPE mixtures. We found that AFA-PLN^{N27A}'s effects on SERCA are the same either in the absence or presence of 20% PE (w/w). In both cases AFA-PLN^{N27A} increases both V_{max} and K_{Ca} (Figure 4). Note that in the presence of AFA-PLN^{N27A} SERCA activity is insensitive to a change in PE content. Therefore, the presence of PLN removes the activating effects of PE on SERCA Ca^{2+} affinity.

PLN Regulation of SERCA and Bilayer Thickness. To analyze how PLN regulation of SERCA depends on the bilayer thickness, we co-reconstituted SERCA and AFA-PLN^{N27A} into bilayers of varied chain length and with 20% (w/w) DOPE to maximize the effects on SERCA activity. From Figure 5, it is clear that the effects of AFA-PLN^{N27A} on SERCA function are dependent on the bilayer composition. In 18:1 bilayers, AFA-PLN^{N27A} binding increases SERCA V_{max} and decreases its Ca^{2+} affinity, as previously noted.[12,23] However, in 22:1 bilayers AFA-PLN^{N27A} does not alter the enzyme's V_{max} but lowers its Ca^{2+} affinity in a manner that is strikingly similar to the behavior of the SERCA/PLN complex in coexpression systems.[11] These results imply that differences in bilayer

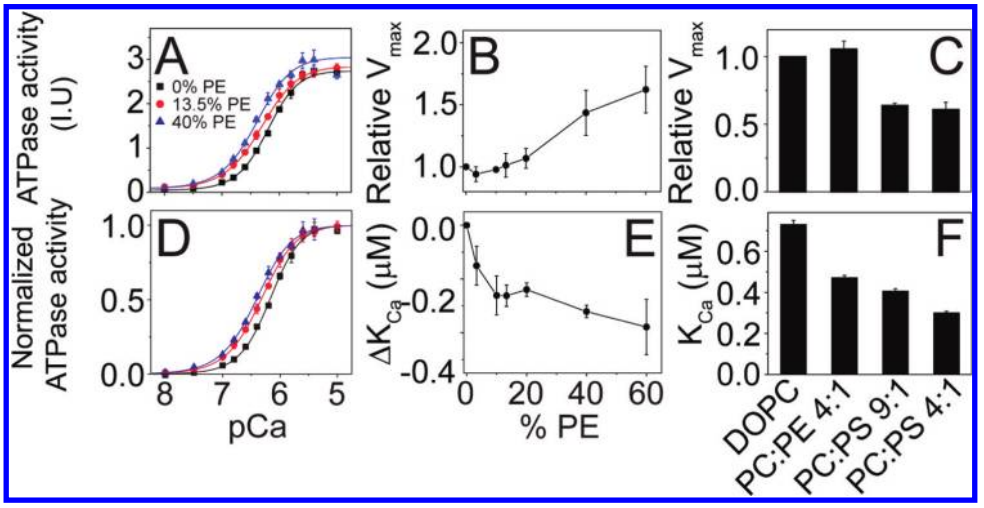

Figure 3. Effects of PE and PS head groups on SERCA activity. (A, D) Representative curves of raw and normalized SERCA activity in bilayers composed of DOPC and different mole fractions of 18:1 DOPE as indicated in figure. Error bars represent an average of triplicate measurements and are for some points smaller than the symbol. (B) V_{max} of SERCA in bilayer with varied amount of PE headgroup. V_{max} values are shown relative to the V_{max} in 0% PE (DOPC). (C) V_{max} relative to $V_{max,DOPC}$ in 4:1 (w/w) DOPC:DOPE and 4:1 or 9:1 (w/w) DOPC:DOPS. (E) K_{Ca} of SERCA in different amounts of PE. ΔK_{Ca} corresponds to $K_{Ca,X\%PE} - K_{Ca,0\%PE}$ where X is the mole fraction of PE in the bilayer. (F) K_{Ca} in DOPC, 4:1 (w/w) DOPC:DOPE and 4:1 or 9:1 (w/w) DOPC:DOPS. Error bars in parts B, C, E, and F represent the standard error of three or more separate reconstitutions. For points where error bars are not visible they are smaller than the symbol.

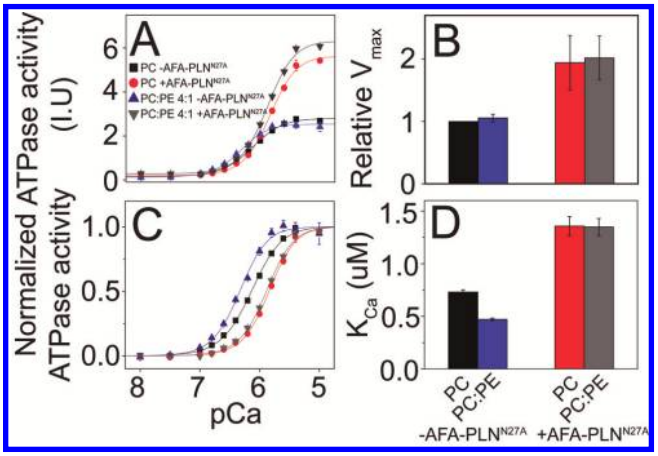

Figure 4. Inhibition of SERCA by AFA-PLN^{N27A} in bilayers with different headgroup composition. (A, C) Representative curves of raw and normalized SERCA activity in DOPC and 4:1 (w/w) DOPC:DOPE in the absence and presence of a 10-fold molar excess of AFA-PLN^{N27A}. Each point shows the average and standard error of three measurements; for some points the error bar is smaller than the symbol. (B) Average V_{max} in DOPC and 4:1 (w/w) DOPC:DOPE in the presence and absence of AFA-PLN^{N27A} Values are reported relative to the value in DOPC. (D) Averaged K_{Ca} values. Error bars in (B) and (D) represent the standard error of three or more separate reconstitutions.

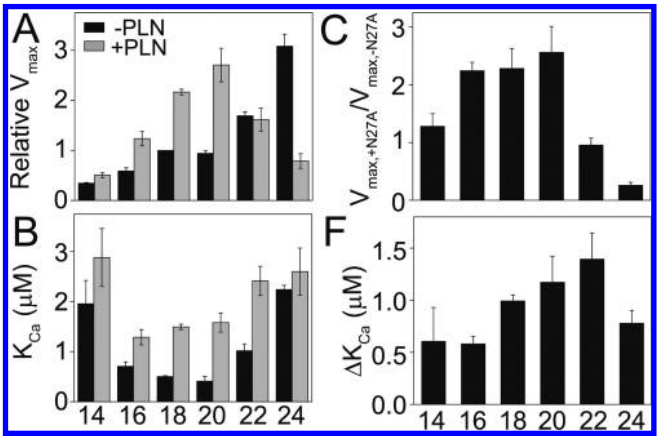

Figure 5. Effects of AFA-PLN$_{N27A}$ on SERCA V_{max} and K_{Ca} in bilayers of varying thickness composed of 4:1 (w/w) 14:1−20:1$^{\Delta 9\text{-Cis}}$ PC:DOPE. (A, B) V_{max} values relative to 4:1 (w/w) DOPC:DOPE in the absence and presence of AFA-PLN$_{N27A}$. (C) SERCA V_{max} change caused by AFA-PLN$_{N27A}$ measured as $V_{max,+AFA\text{-}PLNN27A}/V_{max,-AFA\text{-}PLNN27A}$. (D, E) K_{Ca} values in the absence and presence of AFA-PLN^{N27A}. (F) K_{Ca} change due caused by AFA-PLN^{N27A} and measured as $\Delta K_{Ca} = K_{Ca,+AFA\text{-}PLNN27A} - K_{Ca,-AFA\text{-}PLNN27A}$. The error bars represent standard error for ≥3 separate reconstitutions.

composition may account for some of the discrepancies seen between *in vitro* and *in vivo* studies. In addition, AFA-PLN^{N27A} shifts the highest V_{max} of SERCA to thinner bilayers (from 24:1 to 20:1). The slight difference in maximum V_{max} in the absence of PLN from that measured in pure PC bilayers (Figure 1) is probably attributable to the DOPE (18:1) lipids, which may reduce the thickness of 20:1, 22:1, and 24:1 bilayers. As shown in Figure 4, AFA-PLN^{N27A} removes the activating effects of PE on SERCA. Also, AFA-PLN^{N27A} attenuates the differences in K_{Ca} found for bilayers of different chain length (i.e., 5-fold vs 1.5-fold difference between 20:1 and 24:1 in the absence and presence of AFA-PLN^{N27A}, respectively). Thus, PLN binding alters the effects of headgroup composition and chain length on SERCA activity.

Lipid Effects on the SERCA/PLN Complex at Physiological Protein-to-Lipid Ratios. A lipid-to-protein ratio of 700:1 is typically used in the current literature for the ATPase assays.[12,32] To determine if the effects of lipids is preserved under more physiological lipid-to-protein ratios, we measured SERCA activity using 150:1 lipid:SERCA molar ratio. We found that under these conditions both V_{max} (35% increase) and K_{Ca} (0.63 ± 0.02 μM vs 0.47 ± 0.01 μM) are slightly higher than in 700:1 bilayers. Figure 6A shows that incorporation of 20% (w/w) PE leads to an increase of V_{max}, which is also seen in 700:1 bilayers but at slightly higher PE

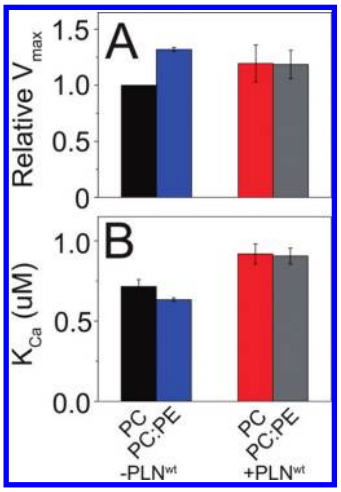

Figure 6. Lipid effects on SERCA and PLN^{wt} at a physiological lipid concentration. SERCA activity in samples reconstituted with a lipid:PLN^{wt}:SERCA molar ratio of 150:5:1. (A) Average V_{max} in the presence and absence of PLN^{wt} in DOPC and 4:1 (w/w) DOPC:DOPE. V_{max} is relative to the value in DOPC. (B) Averaged K_{Ca} values. All error bars represent the standard error of three separate reconstitutions.

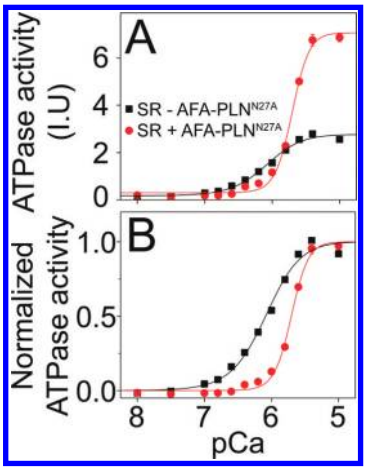

Figure 7. SERCA and PLN in native lipids. SERCA ATPase activity and AFA-PLN^{N27A} regulation with proteins reconstituted into lipids extracted from rabbit skeletal muscle SR. (A) Raw activity. (B) Normalized values. Error bars display the standard error of three measurements and are smaller than the symbol for several of the data points.

concentrations. Also, 20% (w/w) PE leads to lower K_{Ca} (Figure 6B) and thus a higher apparent Ca^{2+} affinity in agreement with the data obtained with 700:1 lipid-to-protein ratio (Figure 3). Therefore, the conclusions obtained in bilayers with a 700:1 lipid:SERCA molar ratio are also valid for 150:1 bilayers, which mimic the SR composition.

In the cardiac SR membrane, SERCA is surrounded by a ~5-fold molar excess of PLN.[38] Wild-type PLN (PLN^{wt}) forms a pentamer[39,40] but de-oligomerizes into monomers to form a 1:1 complex with SERCA.[41,42] To supplement the results with the superinhibitory, monomeric AFA-PLN^{N27A} mutant, we co-reconstituted PLN^{wt} with SERCA at a 5:1 PLN^{wt}:SERCA molar ratio into bilayers containing 150 lipid molecules per SERCA, mimicking the conditions of the native SR membrane. As for the superinhibitory monomeric PLN, addition of PLN^{wt} increases K_{Ca} and raises V_{max} of SERCA (Figure 6). Importantly, in the presence of PLN^{wt}, PE head groups do not have an effect on the K_{Ca} or V_{max} of SERCA in analogy to the results with AFA-PLN^{N27A} at a 700:1 lipid:SERCA molar ratio (Figure 4). Thus, we conclude that the effects of lipids are similar for pentameric and monomeric PLN and that our results with AFA-PLN^{N27A} are representative also for other PLN variants.

SERCA Function and Regulation by PLN in Native Lipids. Previous studies have shown discrepancies in SERCA function and PLN regulation (V_{max}) between different membrane mimicking systems.[11,12,23] To confirm that our conclusions in model membranes apply to native SR lipids, we reconstituted SERCA and AFA-PLN^{N27A} into lipids extracted from rabbit hind-leg skeletal muscle. While these extracted lipids cannot fully mimic all properties of the SR membrane (i.e., asymmetric distribution of lipids between bilayer leaflets or presence of lipid rafts), they allowed us to test SERCA activity and PLN regulation in a fully physiological bilayer composition. In SR lipids, SERCA V_{max} ($V_{max,SR}/V_{maxDOPC}$ = 1.3) and K_{Ca} (0.81 μM) are slightly higher than in the 4:1 (w/w) DOPC:DOPE bilayers used to mimic the SR composition (Figure 7). Addition of AFA-PLN^{N27A} leads to an increase in V_{max} ($V_{max,+PLN}/V_{max-PLN}$ = 2.6) and a shift in K_{Ca} (to 2.0 μM). These effects are similar to those obtained in synthetic lipid preparations (Figure 4). Interestingly, addition of AFA-PLN^{N27A} increases the degree of Ca^{2+} binding cooperativity (Hill coefficient). This effect has been seen in SR vesicles[43] and reconstituted lipid bilayers consisting of a mixture of natural egg yolk PC and PA (phosphatidic acid) lipids,[23] but was not detected in synthetic model membranes (Figure 4). Nevertheless, the differences between the native lipids and the DOPC:DOPE bilayers are relatively minor considering the sensitivity of SERCA to the bilayer thickness and the nature of the lipid headgroup.

DISCUSSION

The importance of lipid bilayer composition for protein function is well established for SERCA[44,45] as well as for other membrane bound enzymes.[46,47] In the present study, we show that SERCA V_{max} and K_{Ca} are significantly affected by the lipid bilayer composition. Thus, lipids with different chain length and head groups have activating or inhibitory effects on the enzyme. Since PE and PS lipids increase the apparent Ca^{2+} affinity of SERCA (Figure 3), we believe these to be *activating* with respect to PC lipids. This activation could be a result either of specific protein−lipid interactions[48] or nonspecific effects on bilayer properties.[49] Previously, it has been shown that addition of PE to PC bilayers does not alter lipid chain dynamics or the overall SERCA rotational correlation time[18] and that change in lipid headgroup composition has a marginal effect on bilayer thickness.[50] Thus, the SERCA activation by PE head groups is likely due to specific protein−lipid interactions rather than macroscopic changes of the lipid bilayer properties. A possible explanation for such interactions is that in contrast to PC, PE and PS can function as hydrogen bond donors and potentially form hydrogen bonds with sites on SERCA, in agreement with other membrane proteins.[48] In fact, lipid molecules have been detected in SERCA crystal structures[3] with the bound lipids modeled as PE and located in the groove between helices M2, M4, and M6. These lipids could possibly be responsible for the activating effects of PE on SERCA. Interestingly, this lipid binding site overlaps with the proposed binding site for PLN.[51] Here, we showed that in the presence of

PLN SERCA activity is independent of PE (Figure 4). Therefore, the inhibitory PLN and the activating PE lipids might compete for the same binding site, which could explain part of the inhibitory effect that PLN has on SERCA.

It is widely accepted that PLN inhibits SERCA by lowering its apparent Ca^{2+} affinity. However, contrasting studies have reported that PLN increases[12,23] or does not affect V_{max}.[11] The increase of V_{max} has mainly been observed in reconstituted systems but vanishes at high lipid:SERCA ratios.[12] Based on our results in model membranes, PLN increases (16:1, 18:1, 20:1), does not change (22:1) or decreases (24:1) the enzyme's V_{max} in bilayers of different thickness (Figure 5). In 22:1 bilayers AFA-PLN^{N27A} lowers the Ca^{2+} affinity of SERCA without affecting V_{max}. The similarity of these data with those from coexpression systems[11] raises the question whether SERCA and PLN preferentially localize to thicker regions of the bilayer *in vivo*. Another possibility is that PLN changes the thickness of the lipid bilayers, affecting V_{max}. In the absence of PLN (and in the presence of 20% (w/w) DOPE), SERCA reaches its maximum activity in 24:1 lipid bilayers (Figure 5A). In contrast, when co-reconstituted with AFA-PLN^{N27A}, SERCA reaches its maximum activity in 20:1 lipid bilayers (Figure 5A). This result may be rationalized by a thickening of the bilayer caused by PLN. Under diluted conditions (high lipid:AFA-PLN^{N27A} molar ratios), the effects of AFA-PLN^{N27A} on V_{max} vanishes.[12] It is possible that under these conditions AFA-PLN^{N27A} concentration is too low to affect the thickness of the bilayer, thus losing its ability to increase V_{max}. In fact, depleting membrane proteins from bilayers can change the thickness by ~5 Å,[52] which corresponds to a difference in chain length of ~3 carbons. In the native SR membrane, the lipid:SERCA molar ratio is ~100:1[53] and thus significantly lower than the 700:1 ratio used in our measurements. Even if additional proteins, lipids, and other biomolecules are present *in vivo*, PLN-induced changes to the lipid bilayer could be important for regulation of SERCA in the muscle cell. Previous studies have shown that the effect of bilayer thickness on SERCA V_{max} is correlated to reversible aggregation.[15] In light of those results, SERCA aggregation is another possible mechanism for regulation of the enzyme by PLN. Also, PLN is in equilibrium between different conformational states,[32,54,55] which are sensitive to the composition of the lipid bilayer.[56,57] Moreover, negatively charged lipids influence the ability of a soluble peptide corresponding to residues 1−23 of PLN to regulate SERCA activity.[58] Further studies are needed to determine if a shift in the conformational equilibrium of PLN is responsible for the effects of lipid composition on the SERCA/PLN complex.

Cardiac and skeletal SR membranes have an average lipid chain length of ~18 and an average of 1.6 unsaturated bonds per lipid chain. They are mainly composed of lipids with PC head groups (~53% (cardiac), ~68% (skeletal)) with smaller amounts of PE (~27%, ~16%), PS (~10%, 10%), and PI (~1%, ~2%) lipids.[17] Our data show that in bilayers with this chain length (Figure 1), fluidity (Figure 2), and headgroup content (Figure 3) SERCA's apparent apparent Ca^{2+} affinity is optimal while V_{max} is higher in thicker, less fluid bilayers. Thus, the SR membrane appears to be tuned to maximize the apparent Ca^{2+} affinity of SERCA. Our data also show that a PE concentration ≥20% (w/w) raises SERCA V_{max}, and K_{Ca} is significantly lowered at PE concentrations of less than 20% (Figure 3). Therefore, at physiological PE concentrations small changes in the PE headgroup content of the SR could significantly alter intracellular calcium cycling. The same is true for changes in bilayer thickness or lipid saturation.

Changes to the bilayer composition in mice have been shown to occur with muscular dystrophy[59] and aging.[60] In addition, diet can also alter the distribution of lipids, as shown in another mouse study where increased levels of unsaturated lipids occurred from *exclusive* fish intake. These mice had lower Ca^{2+} uptake into the SR.[61] In agreement with this finding, our activity assays using model membranes show that SERCA V_{max} is significantly lower in polyunsaturated 18:$2^{\Delta 9,\Delta 12\text{-Cis}}$ PC than in monounsaturated DOPC (Figure 2). This implies that differences in SERCA function between bilayers with different lipid chain structure could be explained by differences in lipid bilayer thickness. Also, a recent study showed that obesity-induced ER stress can significantly raise the PC:PE ratio of the ER.[62] Interestingly, this change in headgroup composition was correlated to a decreased SERCA activity which is in excellent agreement with our *in vitro* studies.

In the cardiac SR, PLN is present at a 5-fold excess molar concentration with respect to SERCA.[38] Our data show that SERCA activity is significantly less sensitive to alterations of the lipid thickness of the PC:PE bilayer in the presence of AFA-PLN^{N27A}. Thus, PLN may have a *buffering effect* for SERCA in response to any changes in membrane structure due to pathology or diet.

In summary, we have shown that SERCA activity and its regulation by PLN are sensitive to bilayer thickness, fluidity, and headgroup content with maximum activity in bilayers that are slightly thicker than the native SR membrane. SERCA's apparent Ca^{2+} affinity, however, is optimized in bilayers mimicking the native SR membrane. The presence of PLN decreases the calcium affinity, removes the activating effects of PE, and attenuates the effects on SERCA activity in lipids of varying chain length. These new findings for Ca^{2+} translocation underline the importance of lipids for SERCA function and its regulation by PLN and could reconcile discrepancies between reconstituted and coexpression systems.

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ABBREVIATIONS

SERCA, sarcoplasmic reticulum Ca^{2+}-ATPases; PLN, phospholamban; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphoserine; $C_{12}E_8$, octaethylene glycol monododecyl ether.

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