Nanodisc-based kinetic assays reveal distinct effects of phospholipid headgroups on the phosphoenzymatic transition of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase

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Sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase catalyzes ATP-driven Ca\(^{2+}\) transport from the cytoplasm to the lumen and is critical for a range of cell functions, including muscle relaxation. Here, we investigated the effects of the headgroups of the 1-palmitoyl-2-oleoyl glycerophospholipids phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylglycerol (PG) on sarcoplasmic reticulum (SR) Ca\(^{2+}\)-ATPase embedded into a nanodisc, a lipid-bilayer construct harboring the specific lipid. We found that Ca\(^{2+}\)-ATPase activity in a PC bilayer is comparable with that of SR vesicles and is suppressed in the other phospholipids, especially in PS. Ca\(^{2+}\) affinity at the high-affinity transport sites in PC was similar to that of SR vesicles, but 2–3-fold reduced in PE and PS. Ca\(^{2+}\) on- and off-rates in the non-phosphorylated ATPase were markedly reduced in PS. Rate-limiting phosphoenzymatic (EP) conformational transition in 0.1 M KCl was as rapid in PC as in SR vesicles, but slowed in other phospholipids, especially in PS. Using kinetic plots of the logarithm of rate versus the square of mean activity coefficient of solutes in 0.1–1 M KCl, we noted that PC is optimal for the EP transition, but PG and especially PS had markedly unfavorable electrostatic effects, and PE exhibited a strong non-electrostatic restriction. Thus, the major SR membrane lipid PC is optimal for all steps and, unlike the other headgroups, contributes favorable electrostatics and non-electrostatic elements during the EP transition. Our analyses further revealed that the surface charge of the lipid bilayer directly modulates the transition rate.

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2 The abbreviations used are: SR, sarcoplasmic reticulum; SERCA1a, adult fast-twitch skeletal muscle sarcoplasmic reticulum Ca\(^{2+}\)-ATPase; SRL, SR lipid; EP, phosphoenzyme; E1P, ADP-sensitive phosphoenzyme; E2P, ADP-insensitive phosphoenzyme; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-ethanolamine; POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine; PG, phosphatidylglycerol; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-cysteine; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.
leol-s-n-glycero-3-phosphocholine bilayer revealed the locations of phospholipids surrounding the crystallized SERCA1a molecules. Many first-layer phospholipids are hydrogen-bonded with amino acid residues, and some phospholipids are anchored by Arg/Lys–phosphate salt bridges and can follow the movements of transmembrane helices, causing local distortions and changes in thickness of the lipid bilayer. Interestingly, exchanging PC with PE does not alter the Ca\(^{2+}\)-ATPase crystal structure (21). In Na\(^{+}\),K\(^{+}\)-ATPase, particular lipids are directly embedded within specific sites of the protein and have a stabilizing and/or activating role (22, 23). Such specific sites are not known in SERCA1a.

Such previous biochemical and very recent structural interactions underscore the necessity to evaluate the contribution of phospholipid headgroups to ATPase function. The advent of nanodisc, which is composed of a phospholipid bilayer defined phospholipid headgroups to ATPase function. The advent of

**Results**

**Properties of nanodisc formed with Ca\(^{2+}\)-ATPase**

SR Ca\(^{2+}\)-ATPase (SERCA1a) was purified by red-agarose column chromatography and reconstituted into nanodisc with various phospholipid components, and then the SR Ca\(^{2+}\)-ATPase-containing nanodisc (CND) was purified by size-exclusion chromatography (Fig. 2). The EP formation activity of SERCA1a eluted at around 11.5–12.0 min (Stokes diameter 10.8–11.8 nm) and corresponded to a major 280-nm protein peak followed by a smaller peak attributable to empty nanodisc (also see below). The CND fraction collected at 11.5–12.0 min shows almost a single band in native PAGE (Fig. 3A) without major impurities, and the empty nanodisc fraction collected at 12.2–13.3 min (corresponding to a Stokes diameter of 8.4–10.0 nm) also shows one major band.

The homogeneity and dimensions of empty nanodiscs and CNDs constructed of PC were further examined by transmission electron microscopy (Fig. 4). The images show that the nanodiscs exist as single particles (Fig. 4, A and B) with Feret diameters for the CND of 14.9 ± 1.5 nm (n = 100) and for the empty nanodisc of 11.9 ± 1.2 nm (n = 100) (Fig. 4C), both values consistent with the homogeneous behavior and Stokes diameters of all nanodiscs in size-exclusion chromatography.

The molar ratio of MSP1D1 to SERCA1a in the CND fraction was determined by SDS-PAGE and Coomassie Brilliant Blue R-250 staining. All of the CNDs with different phospholipids contained only SERCA1a and MSP1D1 (two bands by SDS-PAGE) without any other proteins (Fig. 3B), and the empty nanodisc had only MSP1D1 protein. The ratio MSP1D1/SERCA1a is 2.0 in CND formed with 1-palmitoyl-2-oleoyl-2-leol-s-n-glycero-3-phospho-L-serine (POPS), an ideal value for a nanodisc molecule (as shown in Fig. 4D), and somewhat larger in CNDs with other lipids. Then as a test sample, the fraction of CND constructed with PC (MSP1D1/SERCA1a molar ratio 2.5) was
subjected to an additional size-exclusion chromatography step (Fig. 3C). The early part of the protein peak shows the ideal molar ratio of 2.0 for MSP1D1/SERCA1a, and the ratio increases in the latter part of the peak, indicating that the higher ratio is due to empty nanodisc without SERCA1a. Such repeat chromatography led to a large loss of CND sample; therefore, we used the 11.5–12.0 min fraction in the first chromatography for functional analysis of Ca\(^{2+}\)-ATPase in nanodisc. Note again that all of the collected CND samples contained only SERCA1a and MSP1D1 proteins without any other major proteins (Fig. 3B) and are thus suitable for following functional analyses of Ca\(^{2+}\)-ATPase embedded in nanodisc.

Native PAGE reveals the difference in surface charge of nanodisc with different lipids

Advantageously, nanodisc (with or without membrane-embedded protein) can be handled as soluble material and can be analyzed by native PAGE without detergent (Fig. 3A). Migration distance depends solely on charge, size, and shape of the nanodisc. Then where the size and shape of the complex are nearly the same with different phospholipids, the migration distance depends solely on charge. The migration distances of CNDs with acidic phospholipids (PG and PS) are much larger than those of CNDs with neutral phospholipids (PE and PC), showing the effect of the negatively charged headgroups of the lipids. The CNDs with PG and with PS migrate more slowly than empty nanodiscs with the respective phospholipid, probably due to the smaller number of phospholipid molecules when Ca\(^{2+}\)-ATPase is present as well as the larger size. However, PC CND and the empty PC nanodisc migrate similarly, so it seems that the increase in size may be compensated for by a negatively charged protein. Empty PE nanodisc migrates farther than empty PC nanodisc, as is the case with PE CND and PC CND (although the difference is very small in the CNDs); this finding indicates that PE in the nanodisc is forced to possess negative charge under the nanodisc intrinsic atmosphere, as also noted previously (28). CND made with lipids obtained from SR vesicles (SRL) exhibits an intermediate migration distance between CND with PC (or PE) and CND with PS, as also seen with empty nanodiscs, consistent with the composition of the lipids of the SR membrane of PC (68%), PS (11%), and PE (16%) (17).
Ca$^{2+}$-ATPase activity

The Ca$^{2+}$-ATPase activity was determined in 0.1 mM KCl under physiological conditions in the presence and absence of Ca$^{2+}$ ionophore A23187, and the turnover rate is shown (Fig. 5A). The activity is highly sensitive to the phospholipid component in CND. In A23187, the activity of PC CND is comparable with that of SR vesicles, and activities with other lipids (PG, PS, and PE) are much lower, especially with PS (<4% of that with PC). SRL CND has high activity, but still almost half of that with PC, consistent with the content of PC at 68% and not 100% (17).

In the absence of A23187, the Ca$^{2+}$-ATPase activity of SR vesicles is inhibited due to back-inhibition by luminally accumulated Ca$^{2+}$. By contrast, the activities of all CNDs are hardly affected by A23187, in agreement with the structural feature of the nanodisc that both the cytoplasmic and luminal sides of SERCA1a embedded in a nanodisc are exposed to the same solution, and thus there can be no Ca$^{2+}$ uptake and no Ca$^{2+}$ gradient formation. This is very different from the situation of SERCA1a embedded unidirectionally in liposomes.

The slight effect of A23187 on activity in CNDs is consistent with A23187 directly influencing the activity of deoxycholate-purified SR Ca$^{2+}$-ATPase (29). It may be possible that such a direct A23187 effect is observed here lipid-dependently with the nanodisc system.

In Fig. 5B, the Ca$^{2+}$-ATPase activity was determined after solubilization with 2 mM C$_{12}$E$_6$, a non-ionic detergent. In this assay, 1 mM POPC was included in the assay solution to mask the effects of lipids originating from CND; the amount of added POPC exceeds that of the CND samples over 100 times. Once the CND sample is solubilized with C$_{12}$E$_6$ in POPC, the activities of all of the CND samples become nearly equal, showing that the reduced activity of SERCA1a in certain CNDs is reversible, even in PS CND with the lowest activity. The Ca$^{2+}$-ATPase activity of solubilized SR vesicles is lower than that of the CND samples, possibly accounted for by an inhibitory factor in native vesicles that is absent from purified Ca$^{2+}$-ATPase.

Ca$^{2+}$ binding

Ca$^{2+}$ affinity at the high-affinity transport sites of Ca$^{2+}$-ATPase (E2 + 2Ca$^{2+}$ ↔ E1Ca$_2$) was estimated by the Ca$^{2+}$ concentration dependence of EP formation (Fig. 6A). The affinity in CNDs formed with PC and with SRL are comparable with that of SR vesicles ($K_d = 0.26 ± 0.01$ μM with Hill coefficient 2.1 ± 0.1 (Table 1)). The affinity in PE CND is somewhat reduced ($K_d = 0.59 ± 0.03$ μM) and rather lower in PS CND ($K_d = 0.92 ± 0.09$ μM). On the other hand, the affinity in PG CND ($K_d = 0.17 ± 0.02$ μM) is slightly higher than that in SR vesicles.

In Fig. 6B, the time course of Ca$^{2+}$ release from the Ca$^{2+}$-bound active E1Ca$_2$ state was assessed by the loss of EP forma-
Effects of lipid headgroups on Ca\textsuperscript{2+}-ATPase

Table 1
Ca\textsuperscript{2+}-binding properties of SERCA1a in CDN with various lipids determined in Fig. 6

| Headgroup | \(K_d\) (\(\mu M\)) | \(n_1\) | \(Ca^{2+}\) off-rate (s\textsuperscript{-1}) | \(Ca^{2+}\) on-rate\(^{a}\) (\(\mu M\textsuperscript{-1} \text{s}^{-1}\)) |
|-----------|-----------------|--------|-----------------|-----------------|
| SR vesicles | 0.26 ± 0.01 | 2.1 ± 0.1 | 0.70 ± 0.09 | 2.74 ± 0.09 |
| SRL | 0.32 ± 0.03 | 2.2 ± 0.2 | 0.28 ± 0.03 | 0.87 ± 0.04 |
| POPC | 0.38 ± 0.01 | 1.9 ± 0.1 | 0.49 ± 0.11 | 1.30 ± 0.11 |
| POPE | 0.59 ± 0.03 | 1.4 ± 0.1 | 0.35 ± 0.06 | 0.59 ± 0.07 |
| POPG | 0.17 ± 0.02 | 2.0 ± 0.3 | 0.52 ± 0.07 | 1.87 ± 0.07 |
| POPS | 0.92 ± 0.09 | 1.4 ± 0.2 | 0.03 ± 0.01 | 0.03 ± 0.09 |

\(^{a}\) Calculated from \(K_d\) and \(Ca^{2+}\) off-rate.

The effects of phospholipids on the rate-limiting \(Ca^{2+}\) release was measured (Table 1). \(Ca^{2+}\) release in PS CND is extremely slow (see Fig. 6B, inset), and that of the other CNDs with SRL, PG, PC, and PE is only slightly slower than that of SR vesicles. The \(Ca^{2+}\) on-rate (\(E2 + 2Ca^{2+} \rightarrow E1Ca_2\)) estimated from the \(Ca^{2+}\) affinity and \(Ca^{2+}\) off-rate, revealed that binding is extremely slow with PS CND, only 1% of that of SR vesicles, whereas it is somewhat slowed in the other CNDs, from 70 to 20% of that of SR vesicles (Table 1).

**EP transition**

The time course of the rate-limiting isomeric conformational transition of EP (\(E1PCa_2 \rightarrow E2PCa_2\)) followed by a rapid \(Ca^{2+}\) release (\(E2PCa_2 \rightarrow E2P\)) was examined in 0.1 M KCl (Fig. 7, A and B). In the experiments, EP is first formed with ATP for 10 s to reach a steady state, and then EP decay is followed by chasing \(Ca^{2+}\) with EGTA. In the steady state under these conditions, EP is mostly ADP-sensitive \(E1P\) (Fig. 7C, open bar); therefore, EP decay represents the rate-limiting \(EP\) transition process (\(E1PCa_2 \rightarrow E2PCa_2\), which is followed by rapid \(Ca^{2+}\) release and \(E2P\) hydrolysis (Fig. 1)). Decay rates of SRL and PC CNDs are comparable with those of SR vesicles. PG and PE CNDs are slower, and PS CNDs markedly are retarded to 2% of the rate of SR vesicles. The effects of phospholipids on the rate-limiting \(EP\) transition and on \(Ca^{2+}\)-ATPase turnover are consistent (cf. Fig. 5A).

We previously found (30) that the plot of logarithm of rate versus the square of mean activity coefficient of solutes (\(\gamma_{\alpha}^{-2}\)) for a reaction of interest gives a linear relationship, thereby dividing the activation energy into two components: electric and non-electric forces. The slope reflects the amplitude of the contribution of the electrostatic energy, and the intercept at \(\gamma_{\alpha}^{-2} = 0\) reflects the non-electrostatic contributions (or steric effects), because in this hypothetical state, electrostatic interactions on the protein surface are completely shielded. We apply this approach to the \(EP\) transition in 0.1–1 M KCl, whereby electrostatic contributions are gradually suppressed.

In Fig. 8, the log(rate) versus \(\gamma_{\alpha}^{-2}\) plot indeed shows a linear relationship for all CDN samples and SR vesicles. The slopes for neutral lipid CNDs (PC and PE) and with SRL are slightly positive (Fig. 8 and Table 2) as with SR vesicles, indicating that electrostatic interactions between SERCA1a protein and the lipid heads, even with neutral lipids, facilitate the isomeric conformational \(EP\) transition. It is also possible that electrostatic interactions within SERCA1a protein needed for rapid \(EP\) transition (30) are favored by these lipids surrounding the protein.

On the other hand, the slopes for PG and PS CNDs are negative, especially with PS, indicating that their negative charge hampers the \(EP\) transition, reflected in the extremely low \(Ca^{2+}\)-ATPase turnover rate (cf. Fig. 5A).

The intercept at \(\gamma_{\alpha}^{-2} = 0\) for PE CND is markedly reduced, indicating that non-electrostatic restrictions come into play with this lipid during the conformational transition (Fig. 8 and Table 2). Thus, the low \(Ca^{2+}\)-ATPase turnover rate with PE, seen above, is due to direct non-electrostatic inhibition of the \(EP\) transition.

**Relationship between effects of electric forces on \(EP\) transition and surface charge of membrane**

To further explore the nature of the electric component of the lipid–protein interaction and possible effects of surface
charge, a mixture of phospholipids was introduced into the CNDs. As seen with representative examples in Fig. 9A, slope decreases with increasing acidic lipid content. The increase in acidic lipid content is confirmed by native PAGE (Fig. 9, B and C).

For PG/PC mixtures, the slope decreases almost linearly with the increase in Rf value (Fig. 9D), indicating that surface charge directly affects the EP transition rate (rather than a specific PG binding effect on SERCA1α). For PS/PC CNDs, the slope values again decrease almost linearly with increases in the Rf value up to ~0.3 (PS content ~0.5) and then decrease sharply. The latter phenomenon, at high PS proportions, suggests a PS-specific effect on SERCA1α, which impedes the transition, in addition to the unfavorable electrostatic interactions described above.

Discussion

Single Ca2+-ATPase molecule in one CND

The properties of nanodisc constructed with PC and two belts of MSP1D1 proteins have been well characterized (26, 27), and the 4.6-nm thickness with acyl chain length of C16–C18 is optimal for SERCA1α activity (31). The inner diameter and bilayer area of the MSP1D1 bundle are about 7.6 nm and 44 nm², respectively (27), which should be sufficient for harboring the transmembrane section of one SERCA1α molecule (~4–5 nm in diameter and 9.5–11 nm² in area) (20), but probably not two. Indeed, we found the MSP1D1/SERCA1α ratio of all our samples to be ~2 or higher (due to the presence of empty nanodisc) and far from the value 1 (expected for two ATPases per nanodisc). The Stokes diameters of the CNDs estimated from the retention time in the size-exclusion chromatography are between 10.8 and 11.8 nm (Fig. 2), and the Feret diameter determined with PC CND (representative of the four phospholipids) in transmission electron microscopy is 14.9 ± 1.5 nm, and this size range is close to the SERCA1α’s long diameter of ~12 nm. Thus, a single SERCA1α molecule is embedded in one nanodisc. The system is eminently suitable for exploring lipid headgroup effects on function.

PC is the major lipid in native SR membrane and optimal for function

CNDs with PC exhibit rapid ATPase activity, high-Ca2+-affinity binding, rapid ATPase activation upon Ca2+-binding, and a relatively fast rate-limiting EP transition. PS, PG, and PE are all inhibitory, except for a small effect of PG on increasing Ca2+-binding affinity. In fact, PC performs as well as the SR lipids themselves, which begs the question as to why small amounts of PS and PE are present at all, obviously not for pump function. The answer may lie in the need to introduce curvature to the reticular membrane (32, 33) and also for PS to possibly bind Ca2+ in the lumen with its carboxyl group (34), as, in fact, a large part (84%) of PS is located in the inner leaflet of SR membrane (17).

It has recently emerged that phospholipids play an intimate role in the dynamics of Ca2+-ATPase pumping, acting both as deformable entities and anchors during the substantial conformational changes among the transmembrane helices (20). Deformability is intrinsic to the acyl chain region through the hydrophobic effect (35). The stabilizing role is through hydrogen bonding and salt linkages, some temporary or shifting, between phosphoryl entities and basic amino acid residues. PC, with its large head of three hydrophobic methyl groups, contributes to deformability, spread, and anchoring, compared with PE, which has a tendency to self-associate and presumably form a tighter mat (36). In the electron density maps (20), there are several salt linkages between basic amino acid residues and phosphoryl groups of first-layer phospholipids, but Arg-324 on the cytoplasmic P domain at its junction with M4 is prominent in linking to one phospholipid in E1PCa2 (E1Ca2-AlF4⁻-ADP crystal), only to release it and join with two others in E2-PO4 crystal. Actually, we have found that the alanine and glutamate substitutions of this critical Arg-324 markedly retard the EP transition (37). The large methyl groups of choline, projecting away from the protein, may allow a more favorable presentation of the phosphoryl groups to the arginine.

Electric forces for rapid EP transition

In our analysis of the electrostatic and non-electrostatic effects on the crucial rate-limiting EP transition, we found favorable electrostatic contributions to the transition with PC and PE, but inhibitory effects with the negatively charged PS and PG. Thus, not only hydrogen bonding and salt phosphoryl-basic amino acid residue linkages are important for the transition, but also the positive charges of the amino and choline entities of PE and PC, respectively. The EP transition is a com-

![Figure 8. Relationship between logarithm of EP transition rate and square of mean activity coefficient γ². A, EP transition rates were determined with SR vesicles and CNDs in various concentrations of KCl as indicated, otherwise as described in Fig. 7, and their logarithms are plotted versus γ². The values presented are the mean ± S.D. (error bars) (n = 3–5). Solid lines (SR vesicles and CND with SR lipids SRL) and dashed lines (other CNDs) show the least-squares fit in a linear regression, and the fitting parameters, the slope and the intercept at γ² = 0, are listed in Table 2.

Table 2

Fitting parameters in the analysis in Fig. 8

| Sample     | Intercept | Slope   |
|------------|-----------|---------|
| SR vesicles| −2.04 ± 0.10 | 1.00 ± 0.20 |
| SRL        | −1.72 ± 0.16 | 0.16 ± 0.33 |
| POPC       | −1.60 ± 0.07 | 0.20 ± 0.14 |
| POPE       | −2.55 ± 0.12 | 0.48 ± 0.26 |
| POPG       | −1.42 ± 0.17 | −0.91 ± 0.34 |
| POPS       | −1.82 ± 0.07 | −2.22 ± 0.14 |

Effects of lipid headgroups on Ca2+-ATPase


Effects of lipid headgroups on Ca$^{2+}$-ATPase

A complicated step involving a large movement of M4 to incline the P-domain and a 90° rotation of the A-domain, concomitant with an opening of the transport sites to the lumen and release of Ca$^{2+}$/H$^{+}$. The Ca$^{2+}$/H$^{+}$ binding sites are located close to the membrane center and include a cluster of acidic amino acid residues. The two Ca$^{2+}$/H$^{+}$ released to the lumen are exchanged for about two H$^{+}$/H$^{+}$, which means that destabilizing negative charges in the transmembrane binding sites appear during the EP transition.

An intriguing possibility is that charges are stabilized by the positive electrostatic charges of the choline moiety operating through the low dielectric of the membrane interior.

The advantageous effect of the positive charge of PC on the EP transition was confirmed by the introduction of negatively charged PS and PG into POPC CNDs. Increasing the negative charge content of the nanodisc proportionately decreased the slope of the log(rate) versus $\gamma_{\mathrm{EP}}$ plot, showing that the stimulating electrostatic effect of PC is exerted through the overall bilayer surface charge. Surface charge then is what is helping to drive the EP transition, possibly through a stabilizing effect on the developing negative charges at the emptying Ca$^{2+}$/H$^{+}$ sites.

Non-electrostatic effects on EP transition

Whereas PC and PE positively influence the EP transition through electrostatic forces, we also found that PE exerts a direct non-electrostatic, probably steric, inhibitory effect on the EP transition when electrostatic influences are quelled. Inhibition of Ca$^{2+}$-ATPase activity by PE has also been observed in reconstituted liposomes with a PE content of >80% (19). The small size of the PE head may allow it entry into a protein crevasse that normally only opens transiently during the transition, thereby inhibiting the step. In fact, in many crystal structures of SERCA1a, PE is bound, although no PE was added during purification and crystallization (7, 38–42). There may be a negative charge component to this steric inhibition, as empty PE nanodiscs migrate further than empty PC nanodiscs in native PAGE (Fig. 3A), signifying more negative charge, which is detrimental according to the effects of PS and PG. It is also possible that the hydrogen bonds between residues of the ATPase protein and lipid heads are disrupted with PE due to its small headgroup.

PS-specific unfavorable electrostatic effects

PS has a carboxyl group at the head, is more negatively charged and distinct from the other lipids, and has the strongest unfavorable electrostatic effects on the steps that involve large conformational changes: the ATPase activation by Ca$^{2+}$ binding ($E_2 + 2\text{Ca}^{2+} \rightarrow E_1\text{Ca}_2$) and the EP transition. Actually, the rates at 0.1 mM KCl under physiological conditions are only 1–2% of the respective ones of SR vesicles. PG, lacking the carboxyl, is much less inhibitory.

Figure 9. Effects of lipid composition in CND on the slope in the log(rate) versus $\gamma_{\mathrm{EP}}$ plot and $R_f$ value in native PAGE. A, the log(EP transition rate) versus $\gamma_{\mathrm{EP}}$ was determined as in Fig. 8 with CND constructed with various molar ratios of a mixture of PG and PC, and representative data are shown. The values presented are the mean ± S.D. (error bars) (n = 3–5). Solid lines show the least-squares fit in a linear regression. B, CNDs constructed with various molar ratios of PG/PC mixture and of PS/PC mixture were subjected to native PAGE. The marker proteins are applied in the left and right lanes (denoted as M) as in Fig. 3A. C, the $R_f$ values thus obtained are shown. D, the analysis as in A was performed with CNDs of PC, PG, and various molar ratios of PG/PC mixture and PS/PC mixture, and the slope in the plot log(rate) versus $\gamma_{\mathrm{EP}}$ was determined and plotted against the $R_f$ value in the native PAGE. The values are the mean ± S.D. (n = 3) for the $R_f$ and the mean ± S.E. (n = 3) for the slope in the plot.
Although it is not possible to pinpoint the sites responsible for such unfavorable interactions, there is a strong possibility that such sites may be located at the lipid–protein interface at the cytoplasmic region of the protein. The cytoplasmic Ca\(^{2+}\) entry path in E\(_2\) + 2Ca\(^{2+}\) → E\(_1\)C\(_{a2}\) is located at the space created around M1 and M2 (42), and the carboxyl group of the PS head could well retard Ca\(^{2+}\) movements in and out of the binding sites. Also, one of the most crucial and dramatic changes at the protein–lipid interface occurs during the EP transition when M1’ is formed by an M1 kink and embedded at the surface in the membrane (modeled with E\(_1\)C\(_{a2}\)A\(_{f}E\_T\_A\_D\_P\_2\_B\_E\_T\_E\_2\)). A structural change that is largely reversed during the E\(_2\) → E\(_1\)C\(_2\) structural change (see Fig. 2 in Ref. 43). Such motions of M1’ may be severely impeded by the PS headgroup. In fact, the M1’ helix has negatively charged and polar residues (Glu-51, Gln-56, and Glu-58) on the upper side facing motions of M1 ATPase activity measurement and Ca\(^{2+}\) could potentially affect activity. However, we performed the log(rate) versus γ\(_2\) plot) retards the EP transition.

### Experimental procedures

**Materials**

The pMSP1D1 plasmid (Addgene plasmid 20061) was purchased from Addgene. Non-ionic detergent octaethylene glycol monododecyl ether (C\(_{12}\)E\(_{8}\)) was purchased from Tokyo Chemical Industry Co. LTD (Tokyo, Japan). POPS was purchased from Avanti Polar Lipids, and other phospholipids were from NOF Corp. (Tokyo, Japan). Lipids were dissolved in buffer containing 10 mM Tris/HCl (pH 7.5) and 100 mM (54 mg/ml) C\(_{12}\)E\(_{8}\) and stored at −80 °C.

### Expression and purification of nanodisc scaffold protein MSP1D1

An expression vector containing the MSP1D1 gene construct was transformed into E. coli BL21-Gold(DE3) cells (26). E. coli culture cells were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside and cultured for 3 h at 37 °C. The cells were harvested by centrifugation and stored at −20 °C. The E. coli cell pellets were resuspended in a lysis buffer (20 mM sodium phosphate (pH 7.4), 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride) and sonicated. The cell lysate was cleared by centrifugation and was loaded onto Hi60 Ni Superflow Resin Column (Clontech). The column was washed sequentially with the following buffers: (i) 40 mM Tris/HCl (pH 8.0), 0.3 M NaCl, and 1% Triton X-100; (ii) 40 mM Tris/HCl (pH 8.0), 0.3 M NaCl, 50 mM cholate, and 20 mM imidazole; and (iii) 40 mM Tris/HCl (pH 8.0), 0.3 M NaCl, 20 mM imidazole. Finally, the MSP1D1 protein was eluted with 40 mM Tris/HCl (pH 8.0), 0.3 M NaCl, and 0.5 M imidazole and dialyzed overnight against 20 mM Tris/HCl (pH 7.5) containing 0.5 mM EDTA at 4 °C.

### Preparation of SR vesicles

SR vesicles were prepared from rabbit skeletal muscle as described (44, 45); all of the methods were carried out in accordance with institutional laws and regulations of the Asahikawa Medical University, and the experimental protocols were approved by the Animal Experimentation Ethics Committee of the Asahikawa Medical University (license 16006).

### SERCA1a purification

SR vesicles (2 mg of protein/ml) were solubilized with 10 mg/ml C\(_{12}\)E\(_{8}\) in 10 mM Tris/HCl (pH 7.5) and 10 mM CaCl\(_{2}\). Subsequently, SERCA1a was allowed to bind to Reactive Red 120 resin column (Sigma-Aldrich). The flow-through fraction of this step, which contains lipids from SR vesicles, was kept and used as SRL solution for reconstitution. The column was washed by 2 column volumes of buffer (10 mg/ml C\(_{12}\)E\(_{8}\), 20 mM Tris/HCl (pH 7.5), and 10 mM CaCl\(_{2}\)). The SERCA1a protein was eluted in 2 column volumes of an elution buffer containing 10 mg/ml C\(_{12}\)E\(_{8}\), 20 mM Tris/HCl (pH 7.5), 10 mM CaCl\(_{2}\), and 0.5 M NaCl. The SERCA1a sample thus purified was desalted and concentrated by a centrifugation with Vivaspin (10,000 molecular weight cutoff; GE Healthcare).

### Reconstitution of purified SERCA1a in lipid bilayer nanodisc

Purified SERCA1a protein (0.5 mg/ml) was incubated with 20 μM MSP1D1 in 1.1 mM phospholipid (POPC, POPE, POPG, or POPS or their mixture), 10 mM CaCl\(_{2}\), 20 mM Tris/HCl (pH 7.5), and 10 mg/ml C\(_{12}\)E\(_{8}\) on ice for 30 min. For reconstitution of SERCA1a in nanodisc with lipids from SR membrane, SERCA1a was incubated with MSP1D1 in SRL solution obtained as above. Then to remove C\(_{12}\)E\(_{8}\), the mixture was incubated with 0.4 mg of BioBeads SM2 (Bio-Rad)/ml at 4 °C for 4 h with gentle agitation. The beads and aggregated proteins were removed by centrifugation (10,000 × g, 4 min, at 4 °C), and the supernatant was passed through a filter (0.45 μm, cellulose acetate). The nanodisc thus constructed as a protein/lipid mixture was subjected to size-exclusion column chromatography.
Effects of lipid headgroups on Ca\(^{2+}\)-ATPase

Osborn (48) as described previously (49). To concentrate. The samples were stored at −80 °C after freezing in liquid nitrogen. The molar ratio of the SERCA1a to MSP1D1 in the samples was analyzed by quantitative SDS-PAGE using 12.5% polyacrylamide gels and ImageJ software. The content of phosphorylation sites was determined essentially according to Barrabin et al. (45).

Polyacrylamide gel electrophoresis

Native PAGE was performed at 100 V with 5% polyacrylamide gels in 50 mM BisTris/HCl (pH 7.0), 0.1 M 6-aminocaproic acid, and 1 mM EGTA with running buffer 50 mM BisTris/HCl (pH 7.0) and 1 mM EGTA. The sample (3–5 μg of protein) in sample buffer 4% glycerol, 0.1 M 6-aminocaproic acid, 10 mM BisTris/HCl (pH 7.0), and 0.01% bromphenol blue was applied. For quantification of SERCA1a and MSP1D1 protein contents, Laemmli SDS-PAGE (46) was performed with 12.5% polyacrylamide gels, and the SERCA1a protein purified by a deoxycholate treatment of SR vesicles (47) and the above purified MSP1D1 protein were applied on the same gel as standards. The gels were stained with Coomassie Brilliant Blue R-250, and the densitometric analysis and determination of \( R_f \) value were performed by ImageJ software.

\( \text{Ca}^{2+} \)-ATPase activity

The rate of ATP hydrolysis was determined with A23187 or C\(_{12}\)E8 at 25 °C in a mixture containing 10 μg/ml protein, 1 mM \([\gamma-\text{32P}]\text{ATP}, 0.1 \text{ M KCl}, 7 \text{ mM MgCl}_2, 50 \text{ mM MOPS/Tris (pH 7.0)}, \) and 0.1 M CaCl\(_2\), or 2 mM EGTA without added CaCl\(_2\) otherwise as described in the figure legends. The reaction was terminated with 0.1 M HCl, and the amount of \(^{32}\text{P}\) released from \([\gamma-\text{32P}]\text{ATP} \) was quantified with digital autoradiography. The \( \text{Ca}^{2+} \)-ATPase activity was obtained by subtracting the \( \text{Ca}^{2+} \)-independent ATPase activity determined in 2 mM EGTA without added CaCl\(_2\), otherwise as above. The turnover rate was calculated with the content of the phosphorylation site in each sample.

EP formation

SERCA1a phosphorylation was performed with 10 μM \([\gamma-\text{32P}]\text{ATP} \) for 10 s at 0 °C in MOPS/Tris (pH 7.0), 7 mM MgCl\(_2\), 10 μM CaCl\(_2\), and 0.1 M KCl, otherwise as described in the figure legends. The total amount of \( \text{EP} \) and the fraction of \( \text{E}_2\text{P} \) were determined with digital autoradiography after separation by 5% SDS-PAGE at pH 6.0 according to Weber and Osborn (48) as described previously (49).

Transmission electron microscopy

Samples were negatively stained using 2% (w/v) uranyl acetate on the carbon-coated copper grid (400 mesh). Images were acquired on a JEM-1010 electron microscope (JEOL) operated at 100 kV with a cMOS camera (TemCam-F416, TVIPS) and a nominal magnification of \( \times 40,000 \). The Feret diameter was determined with ImageJ software.

Miscellaneous methods

Protein concentrations were determined by the method of Lowry et al. (50) or absorbance at 280 nm. Data were analyzed by nonlinear regression using the program Origin (Microcal Software, Inc.).

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