Biochimica et Biophysica Acta 1788 (2009) 559–566

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Cytoplasmic residues of phospholamban interact with membrane surfaces in the presence of SERCA: A new role for phospholipids in the regulation of cardiac calcium cycling?

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ARTICLE INFO

Article history:
Received 22 August 2008
Received in revised form 10 October 2008
Accepted 29 October 2008
Available online 14 November 2008

Keywords:
Phospholamban
Sarco(endo)plasmic calcium ATPase
Nuclear magnetic resonance
Isothermal titration calorimetry

ABSTRACT

The 52-amino acid transmembrane protein phospholamban (PLB) regulates calcium cycling in cardiac cells by forming a complex with the sarco(endo)plasmic reticulum calcium ATPase (SERCA) and reversibly diminishing the rate of calcium uptake by the sarcoplasmic reticulum. The N-terminal cytoplasmic domain of PLB interacts with the cytoplasmic domain of SERCA, but, in the absence of the enzyme, can also associate with the surface of anionic phospholipid membranes. This work investigates whether the cytoplasmic domain of PLB can also associate with membrane surfaces in the presence of SERCA, and whether such interactions could influence the regulation of the enzyme. It is shown using solid-state NMR and isothermal titration calorimetry (ITC) that an N-terminally acetylated peptide representing the first 23 N-terminal amino acids of PLB (PLB_{1-23}) interacts with membranes composed of zwitterionic phosphatidylcholine (PC) and anionic phosphatidylglycerol (PG) lipids in the absence and presence of SERCA. Functional measurements of SERCA in sarcoplasmic reticulum (SR) vesicles, planar SR membranes and reconstituted into PC/PG membranes indicate that PLB_{1-23} lowers the maximal rate of ATP hydrolysis by acting at the cytoplasmic face of the enzyme. A small, but statistically significant, reduction in the inhibitory effect of the peptide is observed for SERCA reconstituted into PC/PG membranes compared to SERCA in membranes of PC alone. It is suggested that interactions between the cytoplasmic domain of PLB and negatively charged phospholipids might play a role in moderating the regulation of SERCA, with implications for cardiac muscle contractility.

1. Introduction

Muscle contraction and relaxation involves a cyclical movement of calcium ions into and out of the cytoplasm of each muscle cell. The major route of calcium removal from the cytoplasm of muscle cells is via the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) to the lumen of the sarcoplasmic reticulum (SR). The cardiac isoform of SERCA (SERCA2a) is regulated by the transmembrane protein phospholamban (PLB) [1], which associates directly with the enzyme and stabilises it in Ca^{2+} free form. PLB exists predominantly as a homopentamer in the SR membrane, but it is believed that the monomeric form of the protein is the principal inhibitory species [2].

At low cytoplasmic calcium concentrations unphosphorylated PLB diminishes the apparent affinity of SERCA for calcium [3], which decreases both calcium transport and ATPase rates at physiological calcium concentrations. Inhibition of SERCA is relieved at elevated calcium levels [4] or after phosphorylation of PLB at Ser-16 and/or Thr-17 in response to β-adrenergic stimulation, which increases calcium uptake by the SR that is necessary for muscle relaxation [5]. The effect of PLB on the maximal activity (V_{max}) of SERCA is unclear, as some studies have reported no significant effect [6] whereas others show a clear reduction [7,8]. Conclusions about the effects of PLB on V_{max} in co-reconstituted or co-expression systems are unreliable because the yields of active SERCA recovered can vary substantially from preparation to preparation even in the absence of PLB. Calcium dependent activity curves for SERCA in the presence and absence of PLB are therefore usually normalised to V_{max} so as to observe the affinity of SERCA for calcium, which is a more reliable measure of the regulatory effect of PLB.

PLB appears to regulate SERCA in part through interactions within the membrane and studies on truncated PLB analogues showed that the transmembrane region alone is sufficient to lower the affinity of SERCA for calcium [9,10]. Less clear is the role of the cytoplasmic domain of PLB, which is N-terminally acetylated in cardiac SR [11].

Abbreviations: SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase; PLB, phospholamban; NMR, nuclear magnetic resonance; ITC, isothermal titration calorimetry; EPR, electron paramagnetic resonance; SR, sarcoplasmic reticulum; AAA-PLB, a full-length, null-cysteine variant of PLB mutated as C36A C41A C46A; PLB_{1-23}, the N-terminal cytoplasmic residues $_{1-23}$ of phospholamban; DOPC, L-α-dioleoylphosphatidylcholine; DOPE, L-α-dioleoylphosphatidylethanolamine; DOPS, L-α-dioleoylphosphatidylserine; DOPG, L-α-dioleoylphosphatidylglycerol; DMPC, L-α-dimyristoylphosphatidylcholine; MAS, magic angle spinning; PI, phosphatidylinositol; $\Delta\nu_{1/2}$, peak width at half height

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doi:10.1016/j.bbamem.2008.10.029

Mutations of polar residues in the cytoplasmic domain of PLB reveal that several residues in this region are essential for the regulatory interaction with SERCA [12–14] but do not confirm that this region alone is inhibitory. Studies on the effects of water soluble peptides representing the N-terminal residues of the PLB cytoplasmic domain have been apparently contradictory and inconclusive. Sasaki and co-workers showed that a peptide PLB_{1-31}, comprising the first 31 N-terminal amino acids of PLB, lowers maximal activity by 40% when present in a 330-fold molar excess over the enzyme and Lee et al. showed that the shorter peptide PLB_{1-25} has a similar effect on V_{max} [15,16]. By contrast, several other studies have found that peptides encompassing all or some of the first 31 residues of PLB have no effect at all on SERCA activity [17–19]. Lee and co-workers have shown that the maximal activity of SERCA is lowered by PLB_{1-25} only when the peptide is N-terminally acetylated [20] and a recent study from this laboratory has shown that N-terminally acetylated PLB_{1-23} inhibits calcium uptake by SR vesicles at peptide concentrations of less than 100 μM [21].

NMR and EPR data show evidence for a dynamic equilibrium in the PLB cytoplasmic domain between a tense state that is ordered and a relaxed state that is dynamically disordered and extended [22]. SERCA perturbs the dynamic equilibrium of the PLB cytoplasmic domain, causing sites around Ala24-Gln26 of PLB to act as a focus for a conformational and/or orientational transition [23]. Further EPR studies of a monomeric mutant of PLB spin labelled at residue 11 showed that the spin label was quite rotationally mobile in the absence of SERCA, but became more restricted in the presence of SERCA [24]. From these observations, it has been proposed that the cytoplasmic domain of PLB associates with the lipid surface, and that association with SERCA induces a major conformational change in PLB in which the cytoplasmic domain is drawn away from the lipid surface by SERCA [22,24]. This theory is supported by the observation that PLB_{1-23} interacts with membrane surfaces in the absence of SERCA, with a preference for negatively charged lipid head groups [25].

This study examines whether N-terminally acetylated PLB_{1-23}, used as a proxy for the PLB cytoplasmic domain, can interact with the surface of membranes containing the lipids phosphatidylcholine (PC) and anionic phosphatidylglycerol (PG) in the presence of SERCA. We also investigate the effect of the peptide on SERCA activity in SR vesicles, planar native membranes and reconstituted into defined lipid bilayers. The results provide further evidence that the cytoplasmic domain of PLB lowers the maximal activity of SERCA and suggest that the inhibitory effect is modulated by interactions between PLB_{1-23} and negatively charged lipid head groups. The results suggest that PLB-membrane interactions may play a physiological role by moderating the uptake of calcium by the sarcoplasmic reticulum.

2. Materials and methods

2.1. Materials

Synthetic NAc-PLB_{1-23} (>95% pure) was purchased from Peptide Protein Research Ltd (U.K.). All other chemicals were purchased from Sigma.

2.2. Preparation of SR microsomes and further purification of SERCA1a

The fast-twitch skeletal muscle ATPase (SERCA1), rather than the cardiac isoform, was examined in this work as sufficient quantities of enzyme could be obtained for analysis and previous studies have shown that the functional properties of SERCA1 and its inhibition by PLB are similar to those of SERCA2a [6,12,26]. SERCA1a was purified from fast-twitch rabbit skeletal muscle according to a method adapted from East and Lee [27]. First, SR microsomes were prepared from 100 g muscle tissue by differential centrifugation. A fraction of the intact SR vesicles was retained for calcium uptake measurements and the remainder was treated with sodium cholate and subjected to density gradient centrifugation to yield membranes containing approximately 100 mg of ~90% pure SERCA1a as characterized by SDS-PAGE on a 10% resolving gel. Protein concentrations were calculated using an adaptation of the Lowry method [28]. Purified SERCA was reconstituted into DOPC, DMPC-d_4/DOPG and DOPC/DOPG membranes using an adaptation of methods described previously [29–31].

2.3. Activity measurements

Specific Ca^{2+}-ATPase activity was quantified as the amount of inorganic phosphate (P_i) liberated upon ATP hydrolysis at 37 °C, as described previously [31]. Free calcium was calculated from a method adapted from Tatulian et al. [32]. Membranous Na^+/K^+-ATPase was purified from pig kidney microsomal membranes and enzymatic activities and protein concentrations were determined as described previously [33,34].

2.4. Preparation of vesicles

Small unilamellar vesicles (SUVs) were prepared from dimyristoylphosphatidylcholine (DMPC) and dioleoylphosphatidylglycerol (DOPG) or from DMPC and dioleoylphosphatidylcholine (DOPC), with the PC lipids in a 2-fold molar excess in both cases. Lipids were prepared in chloroform and then dried under argon and high vacuum, before resuspension in 10 mM Tris, 1 mM EDTA, pH 7.4. Sonication was carried out on ice, using a Dawe Ultrasonic probe sonicator at 50% duty cycle for 1–2 min, output control 5, to promote formation of SUVs.

2.5. Solid-state NMR

Measurements were performed using a Bruker Avance 400 spectrometer operating at a magnetic field of 9.3 T. The experimental temperature was 37 °C. Samples were confined within the centre of a 4 mm external diameter zirconium rotor. Wide line ^2H NMR spectra of membranes containing DMPC-d_4 were obtained using a double-tuned magic-angle spinning probe without sample spinning. Spectra were recorded as a result of accumulating 60,000 transients with a 1-s recycle delay. The quadrupole echo sequence (90_x–τ–90_y–τ– *acquisition*) [35] was used with a 90° pulse length of 4 μs and inter-pulse delay τ of 22 μs. For ^{31}P magic-angle spinning (MAS) NMR experiments, samples were spun at 4 kHz, maintained automatically to within ±1 Hz. A ^{31}P 90° excitation pulse length of 4.0 μs was followed by sampling of the free-induction decay with simultaneous proton decoupling at a field of 65 kHz. Each spectrum was the result of accumulating 4096 transients with a 2-s recycle delay between scans, giving a total acquisition time of 2.3 h per spectrum.

2.6. Isothermal titration calorimetry (ITC)

Heat flow resulting from peptide binding to lipid vesicles was measured using a high-sensitivity VP-ITC MicroCalorimeter (MicroCal LLC, Northampton, MA), with a reaction cell volume of 1.4448 ml and total injection volume of 279.5 μl. All experiments were performed at 25 °C, at a power reference setting of 15 μcal/s with stirring at 307 rpm. Prior to use all solutions were degassed under vacuum. Data analysis was carried out using the Origin v.7 software developed for MicroCal. Experimental conditions were designed following established protocols [36,37].

The reaction cell contained a 25 μM solution of PLB_{1-23}, in 10 mM Tris; 1 mM EDTA, pH 7.4. A 10 mM suspension of 2:1 DMPC/DOPG or DMPC/DOPC SUVs was prepared in the same buffer and injected via the syringe. The reference cell contained dH_2O. SUVs were titrated in to the peptide solution at intervals of 10 min in 10 μl aliquots, following an initial 3 μl aliquot, and continuing until the syringe was

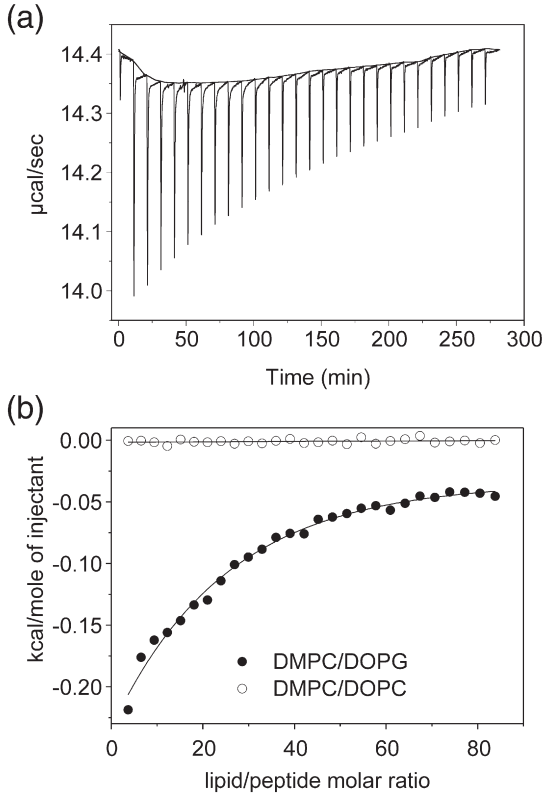

Fig. 1. ITC experiment investigating the relative affinity for membrane binding of PLB_{1-23} to lipids with either a net negative or neutral charge. (a) Titration of 10 mM SUVs of DMPC/DOPG into 25 µM PLB_{1-23}. (b) Curve fit of integrated peaks following titration of DMPC/DOPG (filled circles) and DMPC/DOPC (open circles) SUVs into PLB_{1-23}.

empty. The first 3 µl aliquot was discarded for analysis purposes as recommended in the MicroCal manual. Each injection generates a heat of reaction h_i, which was determined by integration of the individual peaks from the heat flow trace. The heat of dilution $h_{d,i,}$ was determined in controls experiments whereby lipid vesicles were titrated in to buffer solution. Subtraction of $h_{d,i}$ values from experimental values allows the determination of heat flow resulting from peptide binding to lipid.

3. Results

3.1. PLB cytoplasmic domain associates with a higher affinity to anionic lipids

ITC was used to compare the affinity of PLB_{1-23} for membranes of different composition and surface charge (DMPC/DOPC and DMPC/DOPG, with DMPC in a 2-fold molar excess). The two membrane samples have an identical mixture of hydrocarbon chains but differ in the polar headgroups. Titration of DMPC/DOPG SUVs into a 25 µM solution of peptide at 25 °C generated an exothermic binding reaction (Fig. 1). The small enthalpy changes occurring with each titration demonstrate that membrane binding is rather weak and incomplete even after 27 titrations of 10 µl aliquots. It is thus not possible to determine the total binding enthalpy or a complete binding isotherm, but apparent saturation occurred at a molar ratio in excess of 80:1 lipid/peptide, with half maximal binding at a lipid/peptide molar ratio of ~25:1. In contrast, titration with zwitterionic lipids (DMPC/DOPC), results in no observable heat of reaction, indicating a lack of detectable peptide binding to neutral membranes. These results follow the trend of membrane affinity measurements on PLB_{1-23} [25] and are consistent with a relationship between the membrane affinity of PLB_{1-23} and the surface charge density of the membrane in the absence of SERCA.

3.2. PLB cytoplasmic domain associates with anionic lipids in the presence of SERCA

Our previous work investigated whether the cytoplasmic domain of PLB interacts with negatively charged membrane surfaces in the absence of SERCA [25]. Membrane surface interactions were detected by observing the ^2H NMR line shapes for multilamellar vesicles of DOPG and a 2-fold molar excess of DMPC with deuterons at the choline α and β positions (DMPC-d_4). Addition of PLB_{1-23} to the membranes decreased the splitting for the α-deuterons and increased the β-deuteron splitting, indicating that the peptide interacts with the membrane surface and perturbs the orientation of the choline headgroup. Here further experiments investigate whether PLB_{1-23} can also associate with the phospholipids of membranes containing SERCA as such interactions may be physiologically relevant.

The ^2H NMR spectrum of pure DMPC-d_4/DOPG membranes exhibits two sets of Pake doublets with quadrupole splittings ($\Delta\nu_Q$) of ~9.3 kHz for the α-deuterons and ~1.5 kHz for the β-deuterons of DMPC-d_4 (Fig. 2a). In membranes containing SERCA the α-deuteron splitting is reduced to 5.7 kHz and the β-deuteron splitting increased to 2.7 kHz. The spectrum also has a narrow central component, possibly from smaller vesicular structures or micelles. The change in

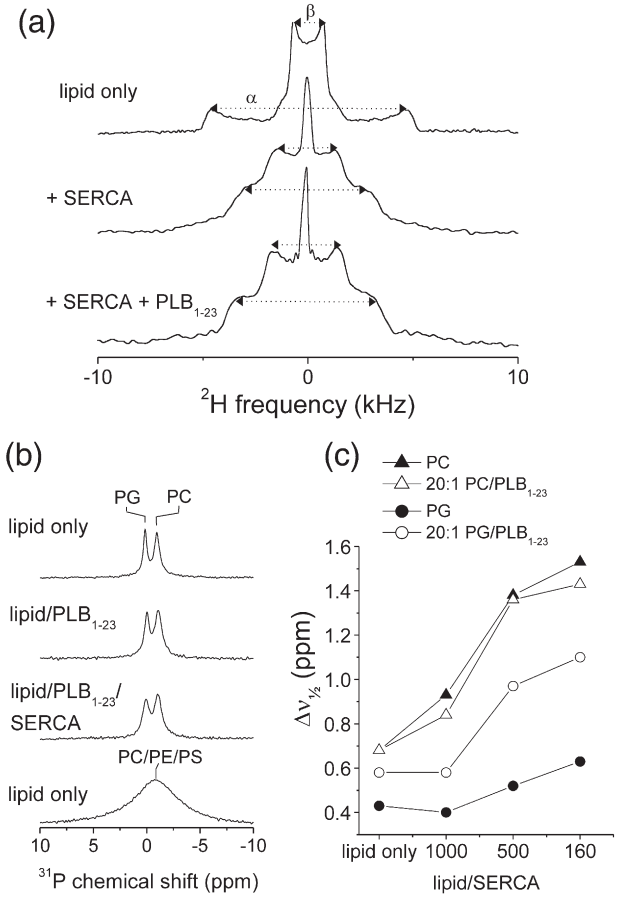

Fig. 2. NMR experiments to examine the interaction of PLB_{1-23} with phospholipid headgroups in membranes containing SERCA. (a) Wide line ^2H NMR spectra of DMPC-d_4/DOPG membranes alone (top), with SERCA at a lipid/protein ratio of 160:1 (middle) and with SERCA and PLB_{1-23} at a lipid/peptide/SERCA molar ratio of 160:8:1 (bottom). (b) A ^{31}P MAS NMR experiment to detect the association of PLB_{1-23} with membrane phospholipids in the absence and presence of SERCA1. Spectra are shown for (from top to bottom): 2:1 DMPC/DOPG membranes alone; DMPC/DOPC membranes with PLB_{1-23} at a *l*/*p* ratio of 20:1; DMPC/DOPG membranes with PLB_{1-23}; SERCA at a lipid/PLB_{1-23}/SERCA ratio of 1000:50:1; and 15:4:1 DOPC/DOPE/DOPS membranes alone. (c) The peak widths at half height ($\Delta\nu_{1/2}$) for DMPC and DOPG in the absence and presence of PLB_{1-23} are shown at different lipid/SERCA molar ratios.

splitting values is consistent with SERCA perturbing the average dynamics and/or orientation of the choline headgroups. The spectrum broadens after the addition of PLB_{1-23} to a lipid to peptide molar ratio of 20:1, with the inner splitting increasing to 3.1 kHz and the outer splitting increasing to 6.5 kHz. These changes are consistent with a further structural rearrangement of the choline headgroups, caused by their association with the peptide. This could occur if the peptide diffuses laterally at the membrane surface or if the peptide associates with specific lipid components and organises them into clusters.

Magic angle spinning (MAS) ^{31}P NMR was used to observe interactions between PLB_{1-23} and individual lipid components of membranes containing SERCA, by exploiting the intrinsic NMR signal from the lipid phosphate head groups. In mixed lipid membranes the association of peptides with the head-groups of specific lipid species can be detected from changes in ^{31}P line widths at half height ($\Delta\nu_{1/2}$) for the different lipids, which generally broaden as a result of motional restriction by the peptide. Spectra were obtained for membranes at different lipid/SERCA ratios in absence and presence of PLB_{1-23}. Experiments were carried out with the DMPC/DOPG membrane system used in the ITC experiments and in our earlier work [25]. Spectra of these membranes alone or in the presence of SERCA and PLB_{1-23} show two clearly resolved peaks for the two lipid components (Fig. 2b, top three spectra). We also examined the effects of PLB_{1-23} on membranes composed of DOPC, dioleoylphosphatidylethanolamine (DOPE) and anionic dioleoylphosphatidylserine (DOPS), which emulate the composition of SR membranes (Fig. 2b, bottom). Unfortunately, none of the individual lipid species could be resolved and so the experiments were aborted. The line widths for the DMPC/DOPG membranes generally broadened as the SERCA/lipid ratio increased, consistent with the laterally diffusing lipids being restrained at the transmembrane interface with SERCA (Fig. 2c). In the presence of PLB_{1-23} at a 20:1 lipid/peptide molar ratio, the line widths for DMPC at the different SERCA concentrations are not significantly different to the line widths in the absence of the peptide. By contrast, at all SERCA concentrations the peak for DOPG is much broader in the presence of PLB_{1-23} than in the absence of the peptide. The selective broadening suggests that the peptide diffuses across the membrane surface and associates preferentially with the DOPG head groups, thereby restraining the anionic lipids even in the presence of SERCA. The ^{2}H and ^{31}P NMR measurements together confirm that PLB_{1-23} associates with lipid headgroups in the presence of SERCA.

3.3. Inhibition of SERCA by null-cysteine PLB

SERCA was reconstituted into dioleoylphosphatidylcholine (DOPC) membranes alone at a lipid/SERCA molar ratio of 500:1, or together with a triple Cys-Ala mutant of PLB (AAA-PLB). The triple mutant of PLB was used as it remains predominantly in the monomeric, SERCA-regulating form [2]. The activity of SERCA alone or in the presence of a 20-fold molar excess of AAA-PLB was measured by observing the amount of inorganic phosphate (P_i) liberated upon hydrolysis of ATP. ATPase activity is coupled to the transport of Ca^{2+} by the enzyme. The normalised calcium concentration-dependent specific activity curve for SERCA in DOPC is shown in Fig. 3. In the presence of PLB the curve shifts to the right signifying a reduction in the affinity of the enzyme for calcium, as reported elsewhere [9,10]. Maximal SERCA activity (V_{max}) also appeared to be depressed in the presence of AAA-PLB (not shown), but as the recovery of active enzyme can vary significantly from sample to sample the effects on V_{max} cannot be confidently attributed to the inhibition of SERCA by AAA-PLB. The role of the cytoplasmic domain in modulating the rate of ATP hydrolysis was investigated further with functional measurements on SERCA in the presence of PLB_{1-23}.

3.4. Inhibition of SERCA by PLB_{1-23}

N-terminally acetylated PLB_{1-23} was previously found to reduce the maximal rate of calcium transport by SERCA (at 1 μM Ca^{2+}) by interacting with the enzyme at the cytoplasmic face, which is accessible to the cytoplasmic domain of full-length PLB *in vivo* [21]. Consistent with this observation, PLB_{1-23} here inhibited ATPase activity by SR vesicles at 1 μM Ca^{2+}. The IC_{50} for ATPase inhibition in SR was approximately 50 μM, although the enzyme remains about 40% active at a peptide concentration of 100 μM (Fig. 4a). These results reflect those of a similar study by Lee and co-workers [15], who showed that PLB_{1-25} inhibits calcium uptake by 40% at a peptide concentration of 50 μM, but disagree with the findings of Jones and Field who did not observe any effect of PLB_{2-25} on calcium uptake by SR vesicles from atrial tumor cells over a 35 min period [18].

The IC_{50} for inhibition of ATPase activity by SERCA in planar membranes occurs at peptide concentrations between 20 μM and 50 μM, with maximal inhibition being about 60% at a peptide concentration of 100 μM (Fig. 4b). PLB_{1-23} does not affect the ATPase activity of Na^+/K^+-ATPase at peptide concentrations of 50 μM and 100 μM (Fig. 4b), consistent with SERCA having an inhibitory binding site for the peptide (and, by implication, the PLB cytoplasmic domain) that is absent in other homologous ATPases. A calcium concentration curve of planar SERCA membranes in the presence and absence of 50 μM PLB_{1-23} demonstrates that the peptide reduces the enzyme V_{max} by about 25% (Fig. 4c). The maximal specific activity of SERCA in planar membranes is reduced from 2.2 μmol P_i/mg/min to 1.6 μmol/mg/min.

3.5. Effect of membrane composition on SERCA inhibition by PLB_{1-23}

Next it was investigated whether the propensity of PLB_{1-23} to associate with phospholipid membranes influences the availability of the peptide to interact with SERCA and regulate enzyme function. The results would suggest whether or not the association of the PLB cytoplasmic domain with SERCA is modulated by the SR membrane environment. This hypothesis was tested by measuring the effect of PLB_{1-23} on the activity of SERCA reconstituted into DOPC membranes with neutral surface charge or DOPC/DOPG membranes with negative surface charge, at a lipid/SERCA molar ratio of 160:1 in both cases. In a 200 μl reaction medium containing 5 μg enzyme, a 50 μM concentration of PLB_{1-23} corresponds approximately to an equimolar peptide/lipid ratio. The ITC results (Fig. 1) suggest that at this ratio a minor fraction of peptide is likely to be bound to the DOPC/DOPG membranes and so any reduction in the inhibition of SERCA compared to that in DOPC is expected to be small. We therefore examined SERCA ATPase activity in the presence of 25–100 μM PLB_{1-23} for three individually reconstituted DOPC/DOPG and three individually reconstituted DOPC samples, each sample measured in triplicate, to observe any small but statistically significant differences in inhibition. The

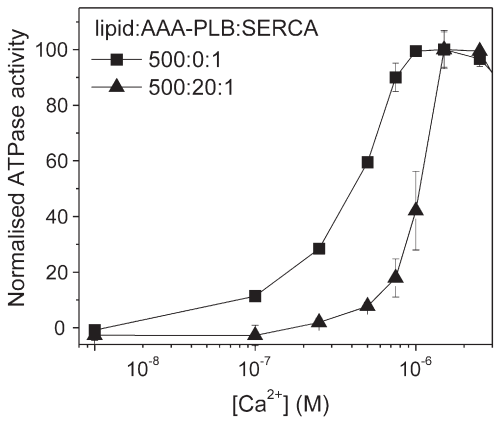

Fig. 3. Calcium-dependent ATPase activity of SERCA1 co-reconstituted with AAA-PLB into DOPC membranes at lipid:AAA-PLB:SERCA1 molar ratios of 500:0:1 (squares) and 500:20:1 (triangles). The curves are normalised to V_{max} for each sample. The maximal activity for SERCA in the absence of AAA-PLB was 4.67 mmol/mg/min.

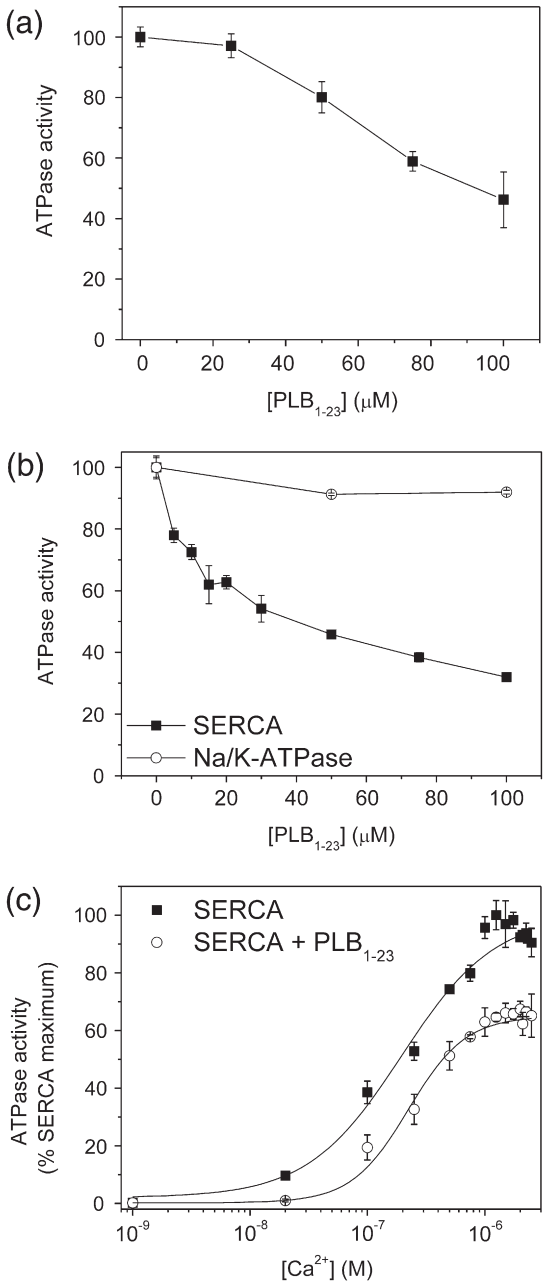

Fig. 4. Analysis of the effects of PLB_{1-23} on SERCA activity. (a) Dose–response curve of ATPase activity of SERCA in SR vesicles at concentrations of up to 150 μM PLB_{1-23}. Activities were measured 3 min after initiation of the reaction by the addition of ATP, with data expressed as a percentage of maximal hydrolytic activity in the absence of peptide (2.6 μmol/mg/min). (b) A dose–response curve of the ATPase activities of purified SERCA (squares) and purified Na,K-ATPase (circles) in planar membranes at concentrations of PLB_{1-23} of up to 100 μM. (c) A calcium curve of the ATPase activity in planar SERCA membranes in the presence (circles) and absence (squares) of 50 μM PLB_{1-23}. Data are expressed as a percentage of maximal hydrolytic activity in the absence of peptide (2.2 μmol/mg/min).

highest PLB_{1-23} concentration (100 μM), which corresponds to a peptide/lipid molar ratio of 2:1, inhibits the ATPase activity of both membranes equally by ~35% (Fig. 5). The lack of distinction at this peptide concentration may be attributed to the large excess of peptide not associated with the membrane. At peptide concentrations of 25 μM and 50 μM, however, there is a significant difference in the inhibition of ATPase activity in the two membrane environments, with inhibition being ~10% greater in the DOPC membranes than in the DOPC/DOPG membranes. Taken together with ITC data, these results indicate that interactions between PLB_{1-23} and membrane surfaces, which occur in the presence of anionic phospholipids, can reduce the availability of the peptide for interacting with the cytoplasmic domain of SERCA. A similar mechanism could be envisaged for the cytoplasmic domain of full length PLB in the SR membrane as will be discussed.

4. Discussion

4.1. The inhibitory effect of the PLB cytoplasmic domain

Much is now understood about how PLB regulates SERCA in the SR membrane, but the precise role played by the 25 or so residues in the cytoplasmic domain of PLB remains a matter of debate. Amino acids within this region associate with SERCA [12–14], but it is not clear whether they act simply to anchor the peptide to its physiological target or whether they contribute directly to SERCA inhibition. It is generally agreed that the PLB cytoplasmic domain is not required for the protein to lower the calcium affinity of SERCA, but how the maximal enzyme activity is affected by the cytoplasmic domain, or, indeed, by full-length PLB, has yet to be confirmed. Hughes and co-workers [7] found that reconstitution of SERCA with a monomeric mutant of full-length PLB lowered both V_{max} and the apparent affinity of the enzyme for calcium, but reconstitution of SERCA with a peptide representing the transmembrane domain of PLB diminished apparent calcium affinity but not maximal activity. Their results imply that the cytoplasmic domain of PLB may be responsible for lowering V_{max}, but alone are not confirmatory.

Measurements of SERCA activity in the presence of water-soluble peptides representing the PLB cytoplasmic domain would seemingly provide a convenient means of answering this long standing question, but results to date have been inconclusive. It is shown here that the peptide PLB_{1-23} decreases V_{max} for ATPase activity of SERCA in SR vesicles, in planar SERCA membranes and reconstituted into defined lipid membranes, but does not lower the affinity of SERCA for calcium. On the other hand, full-length PLB lowers the affinity of SERCA for calcium [9,10], suggesting that the cytoplasmic and transmembrane domains act in concert to regulate enzyme function.

Our results for the functional effect of PLB_{1-23} agree closely with the effects seen for similar peptides by Tada et al. (PLB_{1-31}) and also by Lee et al. who found that the peptide PLB_{1-25} lowers V_{max} without affecting calcium affinity, and shifts the conformational equilibrium of SERCA toward the E1 form. Our results apparently disagree with those of Karim et al. ($PLB_{1-25}K$) and Stokes et al. (PLB_{1-31}), who report that the peptides have no effect on SERCA activity. In the work by the

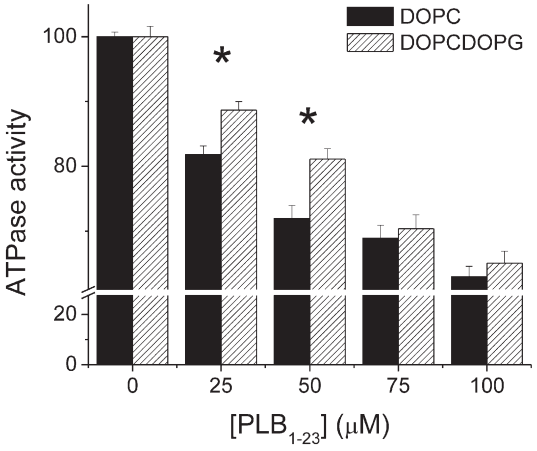

Fig. 5. The effect of PLB_{1-23} upon the rate of ATP hydrolysis by SERCA1 in different membrane environments. Rates of ATP hydrolysis (at a free Ca^{2+} concentration of 1 μM) were measured at 37 °C over a range of PLB_{1-23} concentrations, for 5 μg SERCA in DOPC membranes (squares) or DOPC/DOPG membranes (circles) at a lipid/SERCA molar ratio of 160:1. The maximal ATPase activity of SERCA in the absence of peptide for DOPC and DOPC/DOPG membranes was 2.1 μmol/mg/min and 1.7 μmol/mg/min respectively.

Stokes group, calcium uptake by SR vesicles was measured at a peptide concentration of 7 μM. Here, similar concentrations of PLB_{1-23} also had little or no effect upon ATPase activity, with significant effects observed only at peptide concentrations higher than 20 μM (Fig. 4a and b). In this case, the discrepancy may thus be related simply to differences in peptide concentration. This explanation could also account for the apparent disagreement with the work from Karim and colleagues, who saw no effect of $PLB_{1-25}K$ when the peptide was present in 10-fold excess over SERCA. In their work, ATPase activity was measured at enzyme concentrations of up to 0.6 μg/200 μl, so the actual concentration of $PLB_{1-25}K$ was no higher than 0.3 μM. Jones and Field, however, saw no effect of the peptide PLB_{2-25} on calcium uptake by mouse SR ventricular SR vesicles even at peptide concentrations as high as 440 μM [18]. In cardiac myocytes PLB is acetylated posttranslationally at the N-terminal methionine, and Lee and co-workers have shown that acetylation of PLB_{1-25} is critical for lowering the maximal activity of SERCA [20], although acetylated full-length PLB does not lower the affinity of the enzyme for calcium [38]. In the study by Jones and Field it appears that PLB_{2-25} was not modified in this way, which might explain the inconsistency with our results, since here PLB_{1-23} was acetylated.

4.2. Physiological relevance and significance

Our work does not offer conclusive evidence that the inhibitory interaction between PLB_{1-23} and SERCA reflects the physiological interaction between SERCA and the PLB cytoplasmic domain in SR membranes, but there are positive indications that this may be the case. The inhibitory interaction between PLB_{1-23} and SERCA must occur at the physiologically relevant (i.e., cytoplasmic) face of the enzyme to be able to lower ATPase activity and calcium uptake by SR vesicles. Moreover, the peptide does not affect Na^+/K^+-ATPase activity, suggesting that SERCA has a specific recognition site for the peptide that is not present in homologous ATPases. The peptide PLM_{38-72}, representing the cytoplasmic domain of the Na^+/K^+-ATPase-regulating protein phospholemman, has no effect on the activity of SERCA at a peptide concentration of 100 μM [21], which indicates that SERCA has some selectivity for PLB_{1-23}.

The inhibitory effect of PLB_{1-23} is rather weak and high peptide concentrations (50–80 μM) were needed to achieve 50% reduction in activity. This concentration represents a ~200-fold excess of PLB_{1-23} over the enzyme, whereas full-length, membrane-embedded PLB affects SERCA activity when in a 20-fold excess over the enzyme (Fig. 3). It is erroneous to compare directly the inhibitory capacity of a water soluble peptide, which diffuses in three dimensions, to the effect of the same sequence when attached to the transmembrane domain and diffusing in two dimensions. There is a higher probability of productive collisions occurring between PLB and SERCA when both proteins are embedded in a membrane than when one or both partners are in the aqueous phase. This argument can be qualified with reference to the hypothesis proposed by Kholodenko et al. to explain why signalling proteins should be recruited to cell membranes [39]: "The dissociation constant K_d required for a certain degree of association of two membrane-linked proteins exceeds that required for the same degree of association of two cytosolic proteins by the ratio V_C/V_M", where in this context V_C is the volume of the aqueous medium and V_M is the membrane surface volume accessible to the cytoplasmic domain of PLB. For the conditions of our functional measurements, the lower limit of V_C/V_M is ~40 and so, by anchoring PLB_{1-23} to the membrane, the concentration required to achieve the same inhibitory effect as the aqueous peptide would be lowered by approximately the same factor. This assumes that the membrane anchor is inert, but the fact that the transmembrane domain of PLB alone associates with SERCA and has an effect upon enzyme function will also enhance the inhibitory effect of the cytoplasmic domain compared to that of PLB_{1-23} in solution.

NMR studies of PLB and PLB_{1-23} in lipid bilayers indicate that the cytoplasmic domain associates with the membrane surface [25] and recent opinion is that the overall structure of the pentameric assembly in lipid bilayers has a pinwheel-like appearance with the cytoplasmic domain parallel with the plane of the membrane [40]. These recent results raise the possibility that the structural and orientational

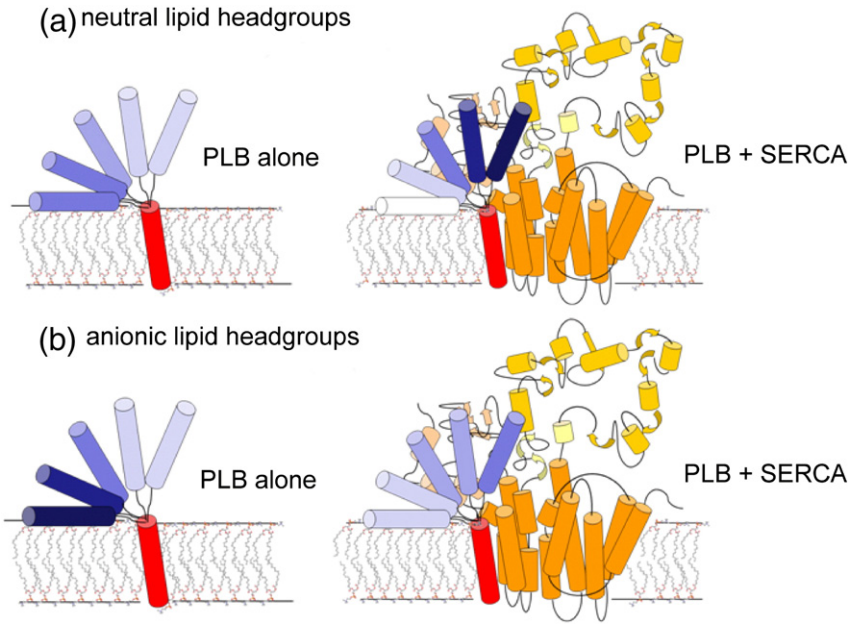

Fig. 6. Schematic of the PLB–SERCA interaction reconciling the structure, dynamics and membrane affinity of the cytoplasmic domain. The monomeric state of PLB is shown for convenience. The cytoplasmic domain of PLB is highly dynamic and its orientational distribution is denoted here by the shade, the dynamic equilibrium being biased toward the darker blue shades. In the absence of SERCA, the PLB cytoplasmic domain interacts transiently with lipid head groups, with higher affinity for negative membrane surfaces (b, left) than for neutral surfaces (a, right). In the presence of SERCA (right) the equilibrium distribution of PLB orientations is displaced toward an upright position allowing contact with the cytoplasmic domain of SERCA. In neutral membranes the cytoplasmic domain of PLB is associated predominantly with SERCA (a, left) but in negatively charged membranes the equilibrium is shifted less toward the SERCA bound orientation and contact with the membrane surface still occurs (b, left). The representation of SERCA is adapted from Ref. [53].

equilibrium of the PLB cytoplasmic domain is influenced by lipid membrane composition and surface charge. Such behaviour is physiologically relevant, because the cytoplasmic face of the SR membrane contains small amounts of the anionic phospholipids phosphatidylserine (PS) and phosphatidylinositol (PI). The structure likely reflects a weighted-average of the PLB cytoplasmic domain orientation, as solid-state NMR measurements have shown it to be highly dynamic and sampling different conformations and/or orientations [41,42].

Here, studies of the effects of PLB_{1-23} on the activity of SERCA reconstituted into membranes of different lipid compositions suggest that the membrane surface charge could moderate slightly the inhibition of SERCA by PLB. Experimental and *in silico* methods have elucidated some of the structural features of the SERCA-PLB complex and our results can be reconciled with previous observations [22,24,43,44] to suggest a model for the role of lipid membranes in the regulation of the complex (Fig. 6). Modelling suggests that the transmembrane domain of PLB can fit into a groove formed by transmembrane helices M4, M6 and M9 of SERCA, allowing K3 in the PLB cytoplasmic domain to extend up toward K397 and K400 in the SERCA cytoplasmic domain some 15–20 Å away from the membrane surface [45]. NMR measurements identified a number of "hot-spots" in the cytoplasmic domain, including basic residues K3, R9, R13 and R14, for which peaks double or disappear in the presence of SERCA, consistent with these residues forming a binding interface with the enzyme [22]. The SERCA-bound and free forms of PLB may therefore have the cytoplasmic domain equilibrium displaced toward either of two extreme (i.e., upright and horizontal) orientations. Interactions between the N-terminal basic residues of PLB and the membrane surface may reduce the propensity of the PLB cytoplasmic domain to adopt the upright orientation necessary to interact with SERCA. In membranes containing only zwitterionic lipids such as DOPC, it is proposed that the dynamic equilibrium is influenced only weakly by the membrane surface (Fig. 6a), but in membranes such as DMPC/DOPG, which have a negative surface charge, the equilibrium is biased further toward a membrane-associated orientation (Fig. 6b). As a result, the fraction of PLB molecules with the cytoplasmic domain in the correct orientation to come into contact with the SERCA cytoplasmic domain is diminished and hence less PLB is available to inhibit the enzyme.

5. Conclusions

It is well established that the phospholipid composition of eukaryotic biomembranes changes with development, diet and disease [46–48]. For example, in diets rich in fish oils the SR membrane adapts to contains less anionic lipids PI and PS. It is thus conceivable that the proportion of anionic lipids in the SR membrane also has an indirect effect on SERCA function mediated by the propensity of the cytoplasmic domain of PLB to interact with the SR membrane surface. Moreover, phosphorylation of PLB, which increases calcium uptake by the SR necessary for muscle relaxation, is associated with partial relief of SERCA inhibition. In the context of the model proposed in Fig. 6, phosphorylation may also alter the orientational equilibrium of the cytoplasmic domain between the SERCA- and membrane-associated extremes.

The interaction between PLB in the cytoplasmic domain and anionic phospholipids raises further questions. The lipid composition of membranes can modulate the function of SERCA enzymes directly, and ATPase activity in bilayers of phosphatidylcholine and anionic phosphatidylserine or phosphatidic acid becomes lower as the fraction of negatively charged lipid increases [49]. Our earlier work suggested that PLB_{1-23} may bind anionic lipids and sequester them into localised clusters, leaving behind pools of predominantly zwitterionic lipids [25]. This raises the intriguing possibility that PLB could influence the local distribution of lipids surrounding SERCA and influence enzyme activity via a secondary mechanism. Moreover, it is noted that most structural studies of PLB using NMR spectroscopy have examined the protein solubilised in micelles of dodecylphosphocholine or incorporated into phosphatidylcholine bilayers, both of which have zwitterionic head groups [50–52]. In the absence of a net surface charge, the micelles and membranes do not necessarily reflect the SR lipid environment and consequently interactions of the PLB cytoplasmic domain with the membrane surface are likely to be weak. By showing that the inhibitory effect of PLB is affected by membrane surface charge, this work draws attention to lipid composition as a consideration when drawing conclusions about the structure–function relationship of PLB *in vitro*.

These results provide motivation for further structural studies of full-length, membrane embedded PLB to investigate the physiological role of the lipid membrane in modulating interactions with SERCA.

Acknowledgements

The British Heart Foundation is acknowledged for Studentship FS/03/090 to J.C.C. and for Project Grant PG/03/162/16425.

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