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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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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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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