Translation of Ser¹⁶ and Thr¹⁷ phosphorylation of phospholamban into Ca²⁺-pump stimulation

Wayne A. JACKSON and John COLYER*

Department of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, U.K.

Stimulation of cardiac sarcoplasmic reticulum Ca²⁺-pump activity is achieved by phosphorylation of the oligomeric protein phospholamban at either Ser¹⁶ or Thr¹⁷. The altered mobility of phosphorylated forms of pentameric phospholamban has been utilized to demonstrate that the mechanisms of phosphorylation of the two sites differ. Phosphorylation of Ser¹⁶ by the AMP-dependent protein kinase proceeds via a random mechanism [Li, Wang and Colyer (1990) *Biochemistry* **29**, 4535–4540], whereas phosphorylation of Thr¹⁷ by calmodulin-dependent protein kinase is shown here to proceed via a co-operative mechanism. This co-operative reaction mechanism was unaffected by the

phosphorylation status of Ser¹⁶. These two mechanisms of phosphorylation generate very different phosphoprotein profiles depending on whether the Ser¹⁶ or Thr¹⁷ residue is phosphorylated. The translation of these patterns of phosphorylation into Ca²⁺-pump function was reviewed using a fluorimetric Ca²⁺-indicator dye, fluo-3, to measure Ca²⁺ uptake by cardiac sarcoplasmic reticulum vesicles. The rate of Ca²⁺ accumulation, which parallels Ca²⁺-pump activity, was stimulated in proportion with the stoichiometry of phospholamban phosphorylation, irrespective of whether phosphorylation was on Ser¹⁶ or Thr¹⁷.

INTRODUCTION

The regulation of sarcoplasmic reticulum (SR) Ca²⁺-pump activity by phospholamban (PLB) is an important control feature of the homeostasis of Ca²⁺ in cardiac muscle. Ca²⁺ ions represent the molecular switch for muscle contraction, and much of the Ca²⁺ required for this purpose is mobilized from the SR in response to an action potential, and returns there to facilitate relaxation. The rate at which Ca²⁺ is sequestered by the SR can be modulated by circulating catecholamines, and this modification contributes to the positive inotropic and chronotropic responses of these agents. Catecholamines modify Ca²⁺ handling primarily through the phosphorylation of PLB. The importance of PLB in the control of cardiac contractility was illustrated recently by the deletion of the PLB gene in transgenic mice [1]. Homozygous PLB[−] mice display cardiac contractile parameters equivalent to PLB⁺ littermates exposed to the synthetic β -agonist, isoprenaline [1]. An equivalent conclusion was made from an earlier study in which monoclonal antibodies to PLB were introduced into cardiac myocytes. The Ca²⁺-handling properties of such cells were stimulated such that a β -agonist could offer no further alteration in the transient [2]. Thus the control of the phosphorylation status of PLB is a major contributor to the contractile status of cardiac muscle.

PLB is a small type-II membrane protein (52 amino acids [3]) associated with the SR/endoplasmic-reticulum Ca²⁺-ATPase (SERCA2) of cardiac muscle, smooth muscle and slow-twitch skeletal muscle [4]. PLB is normally a repressor of SR Ca²⁺-pump function, but phosphorylation by cAMP-dependent protein kinase (PKA) (of Ser¹⁶ exclusively [5,6]) or by calmodulin (CaM)-dependent protein kinase (of Thr¹⁷ exclusively [5,6]) reverses this effect. The relative importance of Ser¹⁶ and Thr¹⁷ phosphorylation in the modulation of cardiac contractility has been difficult to assess. Both sites contribute to the increased Ca²⁺-sequestration rate on β -agonist exposure *in vivo* [7], although the

usage of these two sites appears to vary with species [8]. In terms of Ca²⁺-pump enzymology, both Ser¹⁶ phosphorylation [9] and Thr¹⁷ phosphorylation produce Ca²⁺-pump stimulation [10]; however, phosphorylation of both sites within a single PLB polypeptide does not result in double stimulation of Ca²⁺-pump function [9]. The effects may appear additive in terms of Ca²⁺ transport *in vivo*, if the population of PLB phosphorylated on Ser¹⁶ differs from that phosphorylated on Thr¹⁷, as has been suggested previously [11]. At a molecular level, the interaction of PLB and the Ca²⁺ pump is known to involve residues 2–18 of the cytoplasmic domain of PLB [12] and 397–402 [13] and elements within 467–762 [14] of SERCA2. This interaction is further complicated by the oligomeric arrangement of PLB (pentamer) which has been defined in SDS [5,15,16], and which we have argued previously predominates in native SR membranes [17]. In native SR membranes a number of Ca²⁺-pump units may interact with each pentamer of PLB. In an effort to determine the number of Ca²⁺ pumps likely to be co-ordinated around a single pentamer of PLB, the relative concentration of the two proteins has been determined in SR membranes and in reconstitution studies. Good agreement exists between these two studies which describe a molar ratio of 2:1 PLB/ATPase in SR membranes [9], and 3:1 in reconstitution systems where maximal Ca²⁺-ATPase control had been achieved [18]. These data are consistent with the idea that two Ca²⁺ pumps interact with separate subunits of PLB within a PLB homopentamer as depicted in Figure 1.

The oligomeric structure of PLB does not appear to be essential for biological function [12], but two potential benefits of the tertiary structure might be (i) to facilitate a co-operative mechanism of phosphorylation and dephosphorylation of the protein, and/or (ii) to translate low levels of pentamer phosphorylation into large increases in Ca²⁺-pump function [9]. To investigate these possibilities, a preliminary investigation of the mechanism of Ca²⁺-pump stimulation by Ser¹⁶ phosphorylation has been reported [9], and the data presented were consistent

Abbreviations used: CaM, calmodulin; PKA, cAMP-dependent protein kinase; PKI, peptide inhibitor of PKA; PLB, phospholamban; SR, sarcoplasmic reticulum; SERCA2, SR/endoplasmic-reticulum Ca²⁺-ATPase; ATP[S], adenosine 5'-[γ -thio]triphosphate.

* To whom correspondence should be addressed.

Figure 1 Schematic representation of PLB- Ca^{2+} -ATPase complex

View perpendicular to the bilayer surface using Ca^{2+} -ATPase and transmembrane helical dimensions from ref [19] assuming the protein ratio described in ref. [9].

with three theoretical models, in which Ca^{2+} -pump stimulation was: (i) proportional to phosphorylation stoichiometry; (ii) a threshold event triggered by the generation of tris-phosphorylated pentamer; or (iii) stimulated in a 'delayed linear' manner, i.e. proportional to phosphorylation stoichiometry beyond a critical value (1 subunit/pentamer). The objective of this current study is to define the translation of Ser^{16} and Thr^{17} phosphorylation of PLB into Ca^{2+} -pump function. This first required description of the mechanism of Thr^{17} phosphorylation as had previously been performed for Ser^{16} phosphorylation [16]. We demonstrate that the CaM kinase II endogenous to SR vesicles exploits the close proximity of five phosphorylation sites within a pentameric unit of PLB and exercises a co-operative reaction mechanism of phosphorylation. This results in two distinct pools of PLB: pentamers that are fully phosphorylated and those that are fully dephosphorylated; a pattern of phosphorylation species radically different from that achieved during Ser^{16} phosphorylation [16]. The translation of these two distinctive patterns of PLB phosphorylation into Ca^{2+} -pump stimulation was shown to be simple, with pump activity increasing in proportion to phosphorylation stoichiometry in each case.

EXPERIMENTAL

Materials

Canine cardiac SR vesicles were isolated as described previously [16]. CaM was purified from bovine testes by the method of Gopalakrishna and Anderson [20]. The catalytic subunit of PKA was purified as described previously [21]. The monoclonal antibody raised against canine cardiac PLB, A1 [22], was purified by Protein G chromatography.

Thiophosphorylation of PLB

Thr^{17} thiophosphorylation by CaM-dependent protein kinase

SR vesicles were thiophosphorylated at a concentration of 1 mg/ml by incubating them at 10 °C in 50 mM histidine/HCl, pH 7, containing 5 mM MgSO_4 , 2 μM CaM, 1 μM PKA inhibitor peptide (PKI, 5–22 amide [23]), 0.2 mM adenosine 5'-[γ -thio] triphosphate (ATP[S]) and 1 μM Microcystin-LR. The Ca^{2+} required for this reaction was that which contaminated our buffer solutions ($\sim 1 \mu\text{M}$). Samples were removed over a 30 min time course, and the reaction was terminated by snap-freezing in liquid N_2 . The samples were either used in fluo-3-based Ca^{2+} -

uptake assays (below) or were thawed in the presence of SDS sample buffer in order to determine the mechanism of phosphorylation by subjecting the samples to SDS/PAGE on 15 % polyacrylamide gels [24] and immunoblotting on to poly(vinylidene difluoride) membrane using a semidry blotting apparatus (Pharmacia). The membranes were stained for PLB using a specific monoclonal antibody, A1, as described previously [16]. The electrophoretic separation of PLB phosphorylation species was used to determine the stoichiometry of thiophosphorylation by the method of Colyer and Wang [9].

Ser^{16} thiophosphorylation by PKA

SR vesicles were thiophosphorylated at a concentration of 1 mg/ml by incubating them at 30 °C in 50 mM histidine/HCl, pH 7, containing 5 mM MgSO_4 , 0.2 mM ATP[γ S] and 1 μM Microcystin-LR, and various concentrations of PKA (50–500 nM). Samples were removed after various incubation times in order to achieve particular thiophosphorylation stoichiometries, and the reaction was terminated after the addition of 1 μM PKI by snap-freezing in liquid N_2 . The samples were used in fluo-3-based Ca^{2+} -uptake assays, and an aliquot of each was used to determine the thiophosphorylation stoichiometry [9].

Dual thiophosphorylation of PLB

SR vesicles were thiophosphorylated at a concentration of 1 mg/ml by incubating them at 30 °C in 50 mM histidine/HCl, pH 7, containing 5 mM MgSO_4 , 0.2 mM ATP[γ S], 100 nM PKA and 1 μM Microcystin-LR for 10 min. PKA activity was terminated by the addition of 1 μM PKI, and the endogenous CaM-dependent protein kinase activity was stimulated simultaneously by the addition of 2 μM CaM. The Ca^{2+} required for this reaction was that which contaminated our buffer solutions ($\sim 1 \mu\text{M}$). Samples were removed over a 30 min time course, and the reaction terminated by snap-freezing in liquid N_2 . The samples were thawed in the presence of SDS sample buffer [24], and the mechanism of phosphorylation was determined as described by Colyer and Wang [9].

Control of the endogenous PKA-like kinase of SR

Purified cardiac SR vesicles were incubated at a concentration of 0.5 mg/ml for 10 min at 30 °C in 50 mM histidine/HCl, pH 7, containing 5 mM MgSO_4 , 25 mM NaF, 1 mM vanadate, 1 mM EGTA, 0.2 mM [γ - ^{32}P]ATP (~ 100 c.p.m./pmol) and various concentrations of PKI. Phosphorylation was terminated by the addition of SDS sample buffer [24]. The samples were boiled for 2 min and subjected to SDS/PAGE, using 15 % polyacrylamide gels [24]. The level of phosphorylation in each sample was determined by autoradiography of the dried gels, and quantified by liquid-scintillation counting of pieces of gel which contained PLB.

Control of the endogenous phosphatase of SR

Purified cardiac SR vesicles were incubated at a concentration of 0.5 mg/ml for 30 min at 30 °C in 50 mM histidine/HCl, pH 7, containing 5 mM MgSO_4 , 0.5 μM PKA and 0.2 mM ATP[S]. The phosphorylation was terminated by the addition of 1 μM PKI and the SR vesicles were incubated in the presence or absence of 3.5 mM sodium pyrophosphate for 30 min at 30 °C. Samples were removed at 5 min intervals over this time course and the reaction was terminated by the addition of SDS sample buffer [24]. The phosphorylation status of PLB in all the samples was determined as described by Colyer and Wang [9].

Ca²⁺-uptake assay

Ca²⁺ uptake by purified SR vesicles was followed using a fluo-3-based assay as described previously [25]. SR vesicles (100 µg of protein) were incubated at 37 °C with 10 mM potassium phosphate, pH 7, containing 3.5 mM sodium pyrophosphate, 120 mM KCl, 10 mM phosphocreatine, 10 µg/ml creatine kinase (8 units/ml), 250 nM fluo-3 (free acid, cell impermeant), 1 µM PKI and, in some experiments, 200 nM staurosporin. Ca²⁺ uptake was initiated after 5 min by the addition of 1.5 mM MgATP, and monitored fluorimetrically (excitation 506 nm, emission 526 nm, slits 5 nm). Minimum and maximum fluorescence values were obtained for each assay by the sequential addition of 1 mM EGTA and 3.5 mM CaCl₂ respectively. Fluorescence data were converted into extravesicular Ca²⁺ concentration using the equation:

$$[Ca^{2+}] = K_d(F - F_{min}) / (F_{max} - F)$$

where F_{min} and F_{max} are the minimum and maximum fluorescence values respectively for each assay, and the K_d is the Ca²⁺ dissociation constant for fluo-3 (determined to be 320 nM in our hands, using binding constants described in [26]). The rate of Ca²⁺ uptake was then determined by taking the tangent to the uptake data at the extravesicular [Ca²⁺] of interest. Maximum stimulation of Ca²⁺-uptake activity generated by inclusion of monoclonal antibody (A1 [27]) was established by incubation of the SR sample (100 µg) with A1 (150 µg) for 5 min on ice before transfer into the assay solution.

RESULTS AND DISCUSSION

Thr¹⁷ phosphorylation of PLB

The oligomeric nature of PLB presents an opportunity for co-operation in the mechanism of phosphorylation and dephosphorylation.

The phosphorylation of one subunit within a pentamer may influence the probability of phosphorylation of the others within that structure. This can be studied as a consequence of the electrophoretic separation of pentameric forms of PLB as a function of the number of subunits phosphorylated [17], which has been used previously to demonstrate that Ser¹⁶ phosphorylation by PKA is a random event whereas dephosphorylation of that phosphoprotein is strictly co-operative [16]. To investigate the mechanism of control of Ca²⁺-pump function reversed by PKA-dependent (Ser¹⁶) or CaM-dependent phosphorylation (Thr¹⁷) of PLB, we sought to establish first the mechanism of phosphorylation of Thr¹⁷, and then to establish the translation of both of these events into Ca²⁺-pump stimulation.

Figure 2 depicts the phosphorylation of PLB (pentamer displayed) by a CaM-dependent protein kinase endogenous to the SR. Phosphorylation was completely dependent on Ca²⁺/CaM and resulted in the exclusive phosphorylation of Thr¹⁷ (confirmed by using phosphoamino acid analysis [28] and phosphorylation-state-specific antibodies to PLB [29], data not shown). A change in the mobility of PLB accompanied phosphorylation, as has been demonstrated previously [16], which facilitated description of the thiophosphorylation of PLB to a stoichiometry of ~2.5 mol/mol of pentamer, accompanied by a distinctive pattern of phosphorylation products in which P₅ pentamers (all five subunits phosphorylated) accumulated without a preceding accumulation of pentamers phosphorylated on fewer subunits (P₁–P₄). The concentration of pentamers phosphorylated on an intermediate number of subunits (P₁–P₄) remained low and constant throughout the reaction (Figure 2B). This is indicative of a co-operative reaction mechanism in which phosphorylation of the first subunit within a pentamer promotes the phosphorylation of all others within that unit. Comparison of the experimental distribution of phosphorylated species of

Figure 2 Time course of thiophosphorylation of PLB by CaM-dependent protein kinase endogenous to the SR

SR vesicles were thiophosphorylated by the endogenous CaM-dependent protein kinase activity as described in the Experimental section. (A) Immunoblot showing the oligomeric form of PLB with the phosphorylation species identified (P₀–P₅ respectively). (B) Densitometric analysis of the oligomeric species of PLB phosphorylated for various times. (C) Comparison of the relative concentration of individual phosphorylation species of PLB after 20 min thiophosphorylation (■) with theoretical prediction by the binomial equation at this point in stoichiometry (2.32 mol/mol of pentamer), (□).

Figure 3 Phosphorylation of Thr¹⁷ after prior phosphorylation of Ser¹⁶

Sequential phosphorylation of PLB by PKA and CaM-dependent protein kinase was performed as described in the Experimental section. The densitometric analysis of the oligomeric species of PLB resolved by SDS/PAGE and immunoblotting is presented.

PLB (P₀–P₅) with mathematical predictions based on a random mechanism [16]. (Figure 2C) for the 20 min time point clearly demonstrates that Thr¹⁷ phosphorylation is not a random event, but proceeds by a co-operative mechanism. The co-operative nature of this reaction is significant for two reasons: first it provides evidence that the oligomeric form of PLB is present in the isolated SR membrane (for CaM kinase II to phosphorylate); and secondly it generates a pattern of phosphorylation species distinct from those generated by Ser¹⁶ phosphorylation [16]. An understanding of the translation of these divergent phosphorylation reactions into Ca²⁺-pump function is clearly important.

Before considering the translation of this distinctive pattern of PLB phosphorylation into Ca²⁺-pump stimulation, we performed a further study to determine whether the co-operative phosphorylation reaction was likely to occur *in vivo*. Exposure of cardiac muscle to a β -agonist results in the phosphorylation of both Ser¹⁶ and Thr¹⁷, with the phosphate incorporation into Ser¹⁶ preceding that of Thr¹⁷ [30]. As such the effects of prior Ser¹⁶ phosphorylation on the mechanism of Thr¹⁷ phosphorylation was investigated. Figure 3 shows the densitometric analysis of an experiment in which PLB was thiophosphorylated on Ser¹⁶ to a point close to full stoichiometry (zero time point of CaM kinase phosphorylation). At this point further Ser¹⁶ phosphorylation was inhibited by the addition of PKI, and Thr¹⁷ thiophosphorylation initiated by the activation of the SR CaM kinase II. As was evident in the previous experiments, phosphorylation of Thr¹⁷ was a co-operative reaction with the accumulation of pentamers in which all five subunits were phosphorylated (on both Ser¹⁶ and Thr¹⁷, P₁₀) without the accumulation of forms with a lower phosphorylation status. The co-operative phosphorylation of PLB pentamers is a robust feature of Thr¹⁷ phosphorylation, unaffected by the phosphorylation status of the neighbouring Ser¹⁶ residue.

The divergent phosphoprotein profile obtained with Ser¹⁶ and Thr¹⁷ phosphorylation of PLB may have distinct properties when translated into Ca²⁺-pump function. During Ser¹⁶ phosphoryl-

ation, the entire PLB pool becomes phosphorylated progressively as the stoichiometry rises. With Thr¹⁷ phosphorylation, however, two pools of PLB pentamer are generated, one that is completely dephosphorylated and one that is completely phosphorylated. At low stoichiometry the former pool is of greater size than the latter, with the ratio shifting as the phosphorylation stoichiometry rises. The interpretation of each of these two patterns of PLB phosphorylation by the Ca²⁺ pump is of interest since it will allow extrapolation from phosphorylation data *in vivo* to SR functional parameters. A previous attempt to delineate this issue for Ser¹⁶ phosphorylation was successful in part [9] and identified three theoretical solutions for the experimental observations. These were consistent with Ca²⁺-pump stimulation (i) proportional to phosphorylation stoichiometry, (ii) being a threshold event triggered by the generation of a tris-phosphorylated pentamer, or (iii) stimulated in a delayed-linear manner, i.e. proportional to phosphorylation stoichiometry beyond a critical value (1 subunit/pentamer). Resolution of which of these three alternatives is correct will require the collection of Ca²⁺-transport data of greater accuracy and reproducibility than was achieved previously [9]. To this end, we have employed a fluorimetric Ca²⁺-uptake assay, using fluo-3 to monitor Ca²⁺-pump activity. In this assay, extravesicular Ca²⁺ is monitored continuously by the dye, and the rate of Ca²⁺ uptake by the SR vesicles can be determined at any extravesicular Ca²⁺ concentration from the tangent to the uptake curve at that point. This assay therefore eliminates one source of error present in alternative Ca²⁺-transport assays, namely those associated with Ca²⁺/EGTA additions, employed to achieve calculated pCa.

Figure 4(A) shows a composite graph which characterizes the assay. On addition of MgATP, uptake of Ca²⁺ by the SR vesicles is observed, a process that can be halted by inhibition of pump function (thapsigargin [31], which is followed by the leakage of Ca²⁺ from the vesicles) or by addition of a Ca²⁺ ionophore (A23187). The net uptake of Ca²⁺ measured in this assay clearly represents a balance between Ca²⁺ uptake and release. If the assay is to be used to determine Ca²⁺-pump parameters, then the component of Ca²⁺ release needs to be unaffected by the phosphorylation status of PLB. Figure 4(B) shows the Ca²⁺-release properties of SR vesicles in which PLB phosphorylated on Ser¹⁶ to four different stoichiometries. In these experiments Ca²⁺ uptake into the SR was followed by Ca²⁺ release once ATPase activity was inhibited (250 nM thapsigargin). The rate of Ca²⁺ release was unaffected by phosphorylation of PLB. We are thus convinced that net Ca²⁺ uptake in this assay provides a sensitive and reliable index of Ca²⁺-ATPase function. Similar arguments are required to validate the use of ⁴⁵Ca²⁺-uptake parameters as a measure of Ca²⁺-pump function, which has gained wide support [27,32,33].

Control of SR kinase and phosphatase activities

In order to investigate the relationship between the phosphorylation status of PLB (at each of the two sites) and Ca²⁺-pump activity, the latter needs to be measured at a range of PLB thiophosphorylation stoichiometries. The stoichiometry of phosphorylation must remain constant during the time course of the assay for the measurement to be useful, and thus any endogenous kinase or phosphatase activity must be removed. The SR has been shown to contain an endogenous type-1 phosphatase [34] along with both PKA-like [16] and CaM-dependent kinase [35] activities.

For SR vesicle samples phosphorylated by PKA (Ser¹⁶), only the phosphatase and PKA-like activities are active. In the absence of CaM (as is the case here) the endogenous CaM kinase activity

Figure 4 Net Ca²⁺ uptake as a measure of Ca²⁺-pump function

Ca²⁺ sequestration by SR vesicles was measured as described in the Experimental section. (A) Uptake was initiated by the addition of 1.5 mM MgATP, and accumulated Ca²⁺ released by the addition of A23187 (12 μM). Ca²⁺ uptake was also inhibited by the addition of 250 nM thapsigargin (Tg, trace a). (B) Ca²⁺-release rates were compared for SR vesicles phosphorylated by PKA to various stoichiometries (expressed relative to PLB pentamer). The data represent means ± S.D. (*n* = 4). Release was promoted by the addition of 250 nM thapsigargin at 80 nM Ca²⁺.

Figure 5 Control of endogenous PKA-like kinase

PLB was phosphorylated by kinase activities endogenous to the SR in the presence and absence of peptide inhibitor PKI as described in the Experimental section. Samples were resolved by SDS/PAGE, and the autoradiograph of PLB monomer (P_L) is presented.

is latent. Figure 5 defines the conditions used to control the PKA-like kinase. Phosphorylation of PLB by this kinase was inhibited by the inclusion of increasing concentrations of the peptide inhibitor PKI, which is based on the PKA-specific heat-stable inhibitor protein [23]. Control of the kinase was ensured at 1 μM PKI. Endogenous phosphatase also required inhibition. Thiophosphorylation of PLB was performed to increase the stability of the phosphoprotein; however, this protection was only partial (Figure 6). The addition of pyrophosphate (3.5 mM), which served as a precipitating anion in the Ca²⁺-uptake assay, provided effective phosphatase control (Figure 6). Thus Ca²⁺-uptake assays for Ser¹⁶-phosphorylated samples were performed

Figure 6 Control of endogenous phosphatase

SR vesicles were thiophosphorylated to 4 mol/mol. Dephosphorylation was initiated by the addition of PKI (1 μM), and the time course followed in the absence (▲) or presence (■) of 3.5 mM pyrophosphate. Data points represent single observations, with the data set being representative of two experiments.

in the presence of 1 μM PKI and 3.5 mM pyrophosphate using thiophosphorylated SR samples to prevent alteration of phosphorylation stoichiometry during the experiment.

With Thr¹⁷ (CaM kinase)-phosphorylated SR vesicles, all three endogenous activities are potentially active because CaM is added in order for CaM kinase to be active. Control of this enzyme activity was effectively achieved by the addition of 200 nM staurosporin (non-selective kinase inhibitor; results not shown) to the assay media. Thus Ca²⁺-uptake assays for Thr¹⁷-phosphorylated samples were performed in the presence of 200 nM staurosporin, 1 μM PKI and 3.5 mM pyrophosphate using thiophosphorylated SR samples.

Ca²⁺-pump stimulation by PLB phosphorylation

Having established the Ca²⁺-uptake assay and the conditions required to maintain the phosphorylation status of PLB, SR samples were thiophosphorylated to a range of stoichiometries and the activity of the Ca²⁺-pump was determined. In previous work the translation of Ser¹⁶ phosphorylation into Ca²⁺-pump stimulation was compatible with three models of coupling [9]. These three models were most dissimilar at low phosphorylation stoichiometries, where models (ii) and (iii) (see the Introduction) both predicted little functional change in Ca²⁺ transport at a phosphorylation of 1 mol/mol of pentamer whereas model (i) predicted 20% of maximal stimulation. Thus by focusing attention on this part of the relationship in particular, the mechanism of translation of PLB phosphorylation into Ca²⁺-pump stimulation can be solved.

Multiple SR samples (*n* = 5) were thiophosphorylated by PKA (Ser¹⁶ site) to stoichiometries of approx. 1, 2.5 and 4 mol/mol, and the precise stoichiometry of each was determined. A Ca²⁺-uptake assay was performed on each sample, and the data are presented as means ± S.D. for each parameter (Figure 7A). These data demonstrate that phosphorylation of PLB is accompanied by a change in Ca²⁺-pump activity in direct proportion to the stoichiometry achieved. This relationship is true for all stoichiometries of Ser¹⁶ phosphorylation, including 1 mol/mol pentamer, and this relationship remained in effect for all measured free [Ca²⁺] (63–800 nM Ca²⁺). More complicated models of coupling phosphorylation to pump activity (threshold; delayed-linear mechanisms) fail to predict this event, and are

Figure 7 Dependence of Ca^{2+} -uptake rate on Ser¹⁶ and Thr¹⁷ phosphorylation of PLB

Ca^{2+} uptake by SR vesicles thiophosphorylated to a range of PLB phosphorylation stoichiometries was measured using fluo-3 as described in the Experimental section. Data at 100 nM free Ca^{2+} are shown for Ser¹⁶-phosphorylated SR vesicles (**A**) and Thr¹⁷-phosphorylated SR vesicles (two separate SR preparations; **B**, **C**) (means $\pm$ S.D.; $n = 4$). Reference samples of monoclonal antibody A1-stimulated (#) SR vesicles (**B**), and SR vesicles phosphorylated at the Ser¹⁶ residue (*) of PLB (**B**, **C**) were included for the assays of Thr¹⁷-phosphorylated SR vesicles. Phosphorylation stoichiometry is expressed relative to PLB pentamer. r = correlation coefficient for linear regression.

thus without foundation. From these data we can conclude that, in canine cardiac SR, each phosphorylation of PLB on Ser¹⁶ contributes equally to pump stimulation.

The effect of thiophosphorylation by CaM kinase II (Thr¹⁷ site) on pump activity was then investigated. Thiophosphorylation at this site was limited to stoichiometries in the range 0–2.5 mol/mol of pentamer, which represents the limit of exclusive Thr¹⁷ phosphorylation. Beyond this point, Ser¹⁶ phosphorylation begins to occur catalysed by an independent SR

kinase. Thus to keep the system simple and review the coupling of Thr¹⁷ phosphorylation to pump activity the range of stoichiometries considered was limited to 0–2.5 mol/mol. Figures 7(B) and 7(C) display the relationship between Thr¹⁷ phosphorylation and Ca^{2+} -pump stimulation measured by this assay using two different SR preparations. In Figure 7(B) two reference samples, PKA-phosphorylated SR (Ser¹⁶; 4 mol/mol pentamer; marked by asterisk) and monoclonal antibody A1 (to represent full stimulation as used previously [9,27,32]) have been included for comparison, whereas Figure 7(C) includes the former marker alone. The results show that Ca^{2+} -pump activity was proportional to the stoichiometry of Thr¹⁷ phosphorylation of PLB. No deviation from this relationship is observed at any phosphorylation stoichiometry or at any free $[\text{Ca}^{2+}]$ (63–800 nM Ca^{2+} ; results not shown). The slope of this relationship was indistinguishable from that linking Ser¹⁶ phosphorylation to pump activity (Figure 7A), as witnessed by comparison with the marker point, PKA-phosphorylated PLB. Thus a simple mechanism of coupling of both Ser¹⁶ and Thr¹⁷ phosphorylation of PLB to Ca^{2+} -pump function exists, despite the considerable difference in the pattern of phosphoproteins generated in each case. The translation of Thr¹⁷ phosphorylation into Ca^{2+} -pump function might be expected to be complicated by the phosphorylation of the Ca^{2+} -pump *per se* [36]. In our experience, this phosphorylation would proceed to a very low stoichiometry (~ 0.005 mol/mol) under the assay conditions employed (thiophosphorylation, pH 7.0, 10 °C) and would therefore contribute little to modulation of Ca^{2+} transport in these experiments.

Two comments can be made from these data. First, the simple mechanism of converting phosphorylation of PLB into Ca^{2+} -pump activation (for both Thr¹⁷ and Ser¹⁶ [5]) justifies extrapolation from phosphorylation stoichiometry to Ca^{2+} -pump parameters *in vivo*. This extrapolation carries a single caveat as a consequence of the observation that Ca^{2+} -pump stimulation in response to phosphorylation of one site is not increased further on phosphorylation of the other residue within the same PLB polypeptide [9]. The incidence of dual phosphorylation introduces an element of redundancy into the relationship between phosphorylation and Ca^{2+} transport, such that the direct extrapolation from phosphorylation to activity need not be accurate in all instances. The electrophoretic mobility change associated with two-site phosphorylation (Figure 2 [9,15,30]) can be used to calculate the extent of dual phosphorylation and thereby correct for the redundancy in this relationship *in vivo*.

The second comment concerns the physical change induced by phosphorylation of PLB which results in Ca^{2+} -pump stimulation. In many studies this is viewed as a physical dissociation of the two proteins [37,38]; however, the current data can be interpreted either in these terms or by an alternative model which assumes interaction of the pump with both phosphorylated and non-phosphorylated PLB; but inhibition of pump function only occurs when complexed with the latter form of PLB. In either case, if we view the pump–PLB interaction as a simple receptor–ligand interaction, then a linear response to changes in ligand concentration (which equates to dephosphorylated PLB concentration) would only be expected at low levels of receptor occupancy by the ligand. This suggests that at the Ca^{2+} concentrations considered in this study (63–800 nM), which are of physiological significance, the pump is not saturated with PLB. As a consequence of this, pump activity is only partially inhibited and can respond to phosphorylation of PLB by an increase in Ca^{2+} -transport rate in proportion to the stoichiometry of phosphorylation achieved.

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