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Calcium/calmodulin-dependent protein kinase II activity is increased in sarcoplasmic reticulum from coronary artery ligated rabbit hearts

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Abstract A protein kinase activity intrinsic to the sarcoplasmic reticulum was studied in normal and hypertrophied rabbit hearts. The relationship between this kinase activity and phospholamban phosphorylation was examined. Calmodulin-dependent kinase II activity was found to be increased in sarcoplasmic reticulum preparations from hypertrophied hearts compared with normal. This was evident by measuring the phosphotransferase activity of the kinase and also by examining phospholamban phosphorylation by electrophoretic band shift analysis. Increased phospholamban phosphorylation by Calmodulin-dependent protein kinase II was dependent on prior phosphorylation by cAMP-dependent protein kinase, indicating potential crosstalk. Specific immunoblot analysis of the rabbit sarcoplasmic reticulum identified the presence of the δ form of calmodulin dependent protein kinase II and showed it to be up-regulated in hypertrophied hearts.

Key words: Sarcoplasmic reticulum; Heart failure; Calmodulin-dependent kinase; Phospholamban; Ca^{2+} regulation

1. Introduction

Ca^{2+} release and re-uptake by the sarcoplasmic reticulum (SR) is tightly regulated in mammalian heart and is critical for systolic and diastolic function. In failing heart these functions are compromised. Alterations in Ca^{2+} uptake in failing heart have been suggested to be a result of altered Ca^{2+} pump (SERCA 2) activity and/or expression [1,2]. Since activity of the pump is regulated by phospholamban (PLB), numerous studies have focused on the properties of this protein and the factors that may govern it. Previous work has demonstrated that PLB can be phosphorylated at two sites, serine 16 by cAMP-dependent protein kinase (PKA) and threonine 17 by calmodulin dependent protein kinase II (CaM kinase II). PLB in the dephosphorylated state acts as an inhibitor of SERCA 2. Phosphorylation by the kinases mentioned above relieves this inhibition and the process of active Ca^{2+} transport into the SR lumen is enhanced.

CaM kinase II is a member of a ubiquitous family of Ca^{2+}/calmodulin regulated enzymes. Four isoforms derived from four closely related genes (α, β, γ and δ) have now been identified [3]. Members of the α and β classes are expressed only in neuronal tissues. However, γ and δ isoforms are also expressed in non-neuronal tissues [4,5]. Previous work on porcine myocardium has suggested that the SR associated kinase which phosphorylates PLB is of the δ family [6].

Although it is largely understood how PLB phosphorylation occurs under normal conditions, little information exists concerning protein phosphorylation in cardiomyopathic conditions and the studies that have been performed to date are often contradictory. Previous work by our group has suggested that in the rabbit coronary artery ligation model there is evidence of increased PLB phosphorylation when compared with sham-operated animals [7]. This effect seems to be evident at both serine and threonine sites as observed with phosphorylation site-specific antibodies. In the present study we have examined the possibility that there is increased kinase activity associated with the SR in our experimental hearts that may account for these observations. We have demonstrated for the first time that SR preparations made from hypertrophic remodelled myocardium adjacent to the infarct site, have increased CaM kinase activity which may, either alone or in co-operation with PKA, lead to increased PLB phosphorylation. Using CaM kinase isoform-specific antibodies, we have identified that δ CaM kinase is prevalent in SR isolated from these rabbit hearts. This may be the isoform responsible for the increased activity observed in these preparations.

2. Materials and methods

2.1. Preparation of animals

New Zealand white male rabbits aged 12 weeks and weighing approximately 3 kg were used for experiments. Animals were anaesthetised with hypnorm and maintained with halothane and nitrous oxide/oxygen. A left thoracotomy was performed and the large circumflex branch of the left coronary artery was identified and ligated. Sham-operated animals went through the same procedure but the artery was not tied. Animals were left for 8 weeks to allow cardiac remodelling to occur. Echocardiography of left ventricular function was assessed and ejection fractions ranged from 67–77% for sham-operated animals and from 28–52% for coronary artery ligated animals, with means of $73.0 \pm 1.3\%$ and $43.1 \pm 2.8\%$ respectively ($n = 14$). These values are very similar to previously published data on this model [8]. In coronary artery ligated animals there was significant haemodynamic dysfunction. There was evidence of hypertrophy indicated by increased heart weights and evidence of congestion since both lung and liver wet weights were increased on post-mortem examination [9]. Overall, these results suggest that the coronary artery ligation model produces a significant degree of heart failure in these animals.

Prior to experiments, rabbits were given a lethal injection of pentobarbital sodium (100 mg/kg) and hearts were rapidly removed and washed several times in modified Krebs solution [150 mM NaCl,

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Abbreviations: HEPES, 4-[2-hydroxyethyl]-1-piperazine-ethanesulphonic acid; DTT, dithiothreitol; PMSF, phenylmethylsulphonyl fluoride; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; MOPS, 3-[*N*-morpholino]propanesulphonic acid

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5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM 4-[2-hydroxyethyl]-1-piperazine-ethanesulphonic acid (HEPES), pH 7.0, 10 mM glucose].

2.2. Isolation of SR

The preparation of SR was based on a previously reported method [10]. The isolated free wall of the left ventricle (1–2 g of tissue) was removed, minced using fine scissors and then homogenised using an ultraturrax (Labortechnik) in ice-cold homogenisation buffer [0.3 M sucrose, 10 mM imidazole, pH 7.0, 30 mM histidine, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μg/ml leupeptin and 100 μg/ml aprotinin). The homogenate was centrifuged to remove large particles and was then further processed by several high speed centrifugation and precipitation steps to yield the crude SR preparation as described previously [7]. This was either stored in precipitation buffer (same composition as homogenisation buffer but with 0.6 M KCl) at −70°C or prepared in assay buffer for immediate use.

2.3. PLB phosphorylation studies

SR preparations (50 μg) were incubated at 30°C for the appropriate times in the presence of 2 mM ATP and presence or absence of added calmodulin and calcium at final (free) concentrations of 0.6 and 1.25 μM, respectively in assay buffer containing protease and phosphatase inhibitors [11]. In experiments examining the effect of dual phosphorylation, preparations were incubated for 2 min with 0.2 μM catalytic subunit of PKA (Boehringer Mannheim) prior to addition of calcium and calmodulin. In experiments where the CaM kinase inhibitor peptide (281–301) (Calbiochem) was used it was added for the indicated times at 20 μM. Reactions were left to proceed for the appropriate times and were terminated by the addition of 4×Laemmli sample buffer (6% SDS, 30% glycerol, 235 mM Tris, pH 6.8, 0.005% bromophenol blue, 8 mM β-mercaptoethanol). These were stored frozen until analysis.

2.4. CaM kinase activity assay

The assay was designed to measure the phosphotransferase activity of CaM kinase II in crude preparations and was based on phosphorylation of specific substrate peptide (KKALRRQETVDAL) by the transfer of γ-phosphate of [γ-^{33}P]ATP (DuPont NEN; 1000–3000 Ci/mmol) by the CaM kinase. Activity was measured in SR preparations made at approximately 2–5 mg/ml in assay dilution buffer (20 mM 3-[*N*-morpholino]propanesulphonic acid (MOPS), pH 7.2, 25 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM DTT, 1 mM $CaCl_2$). The final protein content in each assay sample was between 20–50 μg. Assay components (from a kit supplied by Upstate Biotechnology) were mixed on ice and reactions initiated by addition of the [^{33}P]Mg^{2+}/ATP (25μCi/100μl). Incubations were performed at 30°C for the required times and reactions stopped by transferring to P81 phosphocellulose paper. These papers were washed ten times in 0.75% phosphoric acid to remove residual radioactivity and twice in acetone to aid drying. The bound phosphorylated substrate was then quantitated by scintillation counting.

2.5. Electrophoresis and immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described [12] using the Novex system with either 10% or 14% Tris-glycine gels for CaM kinase and PLB detection, respectively. As with previous studies by our group, samples prepared for electrophoresis were not heated and hence, there was no degradation of the pentameric 25 kDa form of PLB into the monomer ~5 kDa. Gels were subjected to electrophoresis at a constant current of 25 mA for 1.25 h for PLB and 1.75 h for CaM kinase. Proteins were transferred to nitrocellulose membranes and these were then blocked in a BSA blocking buffer [7]. Blots were incubated overnight at 4°C with mouse anti-PLB monoclonal antibody A1* (0.0625 μg/ml) and/or PT-17 (1:5000 dilution) (Phosphoprotein Research) or with rabbit anti-δ CaM kinase (0.5 μg/ml) (gift from Dr P. Karczewski, Berlin) or goat anti-γ CaM kinase (0.05 μg/ml) (Santa Cruz). Membranes were washed and then incubated for 2 h at room temperature with either goat anti-mouse IgG-horseradish peroxidase conjugate diluted 1:2000 (Transduction laboratories), goat anti-rabbit IgG-horseradish peroxidase conjugate diluted 1:5000 (Jackson ImmunoResearch)or mouse anti-goat IgG-horseradish peroxidase conjugate diluted 1:4000 (Santa Cruz), for PLB and CaM kinase δ and γ, respectively. The blots were then washed a further three times and developed using the ECL detection system (Amersham).

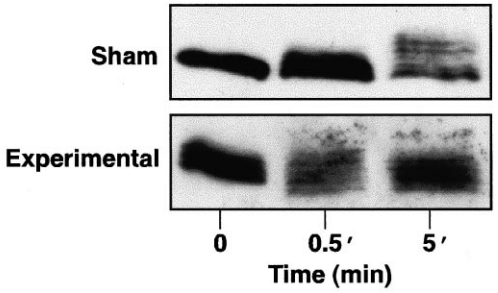

Fig. 1. Comparison of intrinsic PLB phosphorylation in experimental and sham-operated preparations. SR preparations were incubated at 30°C for the indicated times in the presence of ATP. Reactions were stopped by the addition of 4×Laemmli sample buffer and 10 μg samples were subjected to electrophoresis as described. PLB was detected using the A1 antibody which detects both phosphorylated and dephosphorylated forms of PLB. This experiment is representative of six others.

2.6. CaM kinase antibody preparation

Anti-δ specific antibodies were raised in rabbits as previously described [13]. Briefly, the antigenic epitope KENFSGGTSLWQNI corresponding to the C terminus for a subclass of isoforms of δ CaM kinase from rat was used. Peptide conjugation and immunisation was performed as previously described [14] and the resulting polyclonal antibodies were affinity purified on peptide affinity columns [15]. Aliquots of the δ antibody were kindly provided by Dr P. Karczewski (Max Delbrück Centre for Molecular Medicine, Robert Rössle Straße 10, Berlin, Germany). Goat polyclonal anti-γ CaM kinase antibodies were raised against an epitope mapping at the C-terminus of γ CaM kinase II of human origin (Santa Cruz).

2.7. Protein measurements

Protein content was determined using the Coomassie Plus protein assay (Pierce) and BSA (0.1–1 mg/ml) as standards.

3. Results and discussion

Initial experiments examining changes in intrinsic phosphorylation levels of PLB using SR preparations were performed as previously described [7]. Upon addition of ATP and using electrophoretic band shift analysis, we observed increased phosphorylation of PLB in experimental hearts over a 5 min period of incubation (Fig. 1). These results indicate the potential existence of elevated kinase activity intrinsic to the SR.

Assessment of CaM kinase II activity associated with the SR was then made using an assay kit (Upstate) designed to specifically measure activity of this enzyme in crude preparations. We examined phosphotransferase activity in fresh SR preparations over a period of 10 min incubation with a specific substrate peptide (see Section 2) and in the presence of inhibitors for PKA and PKC. Results indicated that CaM kinase activity was higher in preparations from coronary artery ligated rabbit hearts compared with sham-operated animals (mean activity over background was 842 ± 186 cf. 346 ± 48 pmol phosphate inc./min/mg protein from four ex-

* The A1 antibody can detect both de-phosphorylated and phosphorylated (P_1–P_{10}) forms of PLB. PT-17 detects the threonine phosphorylated form of PLB.

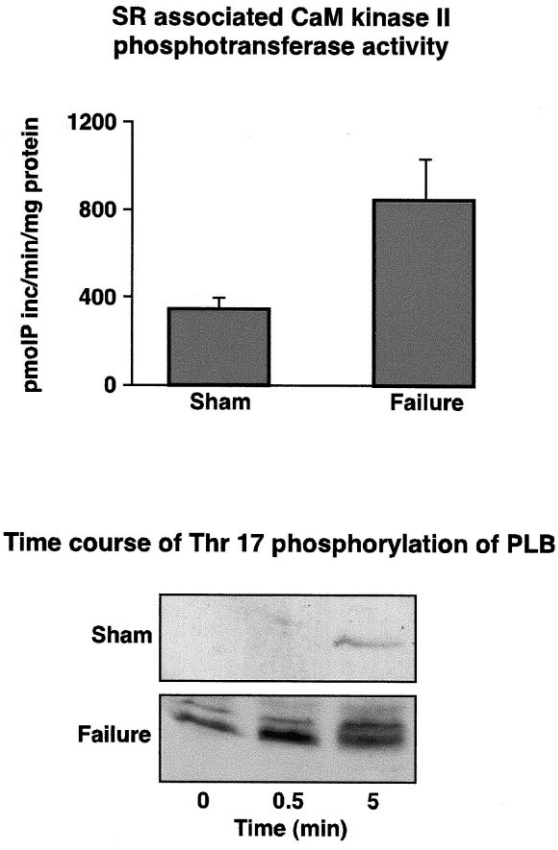

Fig. 2. Measurement of CaM kinase II activity associated with the SR and comparison with Thr-17 phosphorylation of PLB. a: Preparations were made from sham-operated and hypertrophied (experimental) myocardium as described. Phosphotransferase activity of CaM kinase II was measured after 10 min incubation with a specific substrate peptide as described in the Section 2. Data represent the mean ± S.E.M. of four sham and four experimental animals. b: The Thr-17 phosphorylated form of PLB was detected using a phosphoamino acid specific antibody (PT-17) against PLB. SR preparations (10 μg) from a time course experiment (as described for Fig. 1.) were subjected to electrophoresis as described. This experiment is representative of four others.

perimental and four sham-operated animals, respectively) (Fig. 2). Moreover, we performed some preliminary experiments to measure activity in crude cell lysates and also found that to be slightly elevated in preparations from experimental animals. Since this assay is linear with time up to 30 min, we did test a number of other incubation times (shorter and longer) and found that the optimal difference in activity was observed between 5–10 min incubation. In parallel with these studies, we also examined whether there was an increase specifically in the threonine phosphorylated form of PLB. These measurements were made using phosphoamino acid specific antibodies against the Thr-17 form of PLB. Results suggest that in experimental preparations, along with an increase in CaM kinase II activity, there is a corresponding increase in threonine phosphorylated PLB (Fig. 2.).

In a further attempt to link this observed increase in CaM kinase activity with a potential increase in PLB phosphorylation, we performed time course experiments on SR preparations under conditions that had been manipulated to favour activation of either PKA or CaM kinase or both. Using band shift analysis which allows observation of up to 10 different phosphorylation states of PLB with the PLB specific A1 monoclonal antibody, we were able to detect changes in the levels of phosphorylation of PLB after selective stimulation of these kinases. In the absence of any added PKA, the effect of added Ca^{2+} and CaM was examined in sham and experimental preparations (Fig. 3a). As observed in our previous studies, the overall levels of phosphorylation achieved by intrinsic kinase activity over time were higher in the experimental preparations. However, the addition of Ca^{2+} and CaM had no obvious additional effect on phosphorylation in either sham or experimental preparations. Since previous studies had indicated the possibility that in some preparations, serine phosphorylation by PKA may be a pre requisite for stimulation of threonine phosphorylation by CaM kinase, we decided to examine this possibility. We tested whether prior phosphorylation with a sub-optimal dose of PKA might boost activation of intrinsic SR CaM kinase activity and lead to further PLB phosphorylation. SR preparations were treated with 0.2 μM PKA for increasing times and compared with preparations that had been pre-treated with PKA for 2 min and then Ca^{2+} and CaM added for increasing periods. Since sub-optimal levels of PKA were added to these preparations, phosphorylation in the presence of PKA alone, was fairly slow (cf. a complete band shift to P_5 was observed when SR preparations were treated for 10 min with 2 μM PKA, unpublished observations). However, when the same preparations were pre-treated with PKA and then had Ca^{2+} and CaM added, there was an obvious increase in the number of phospho-

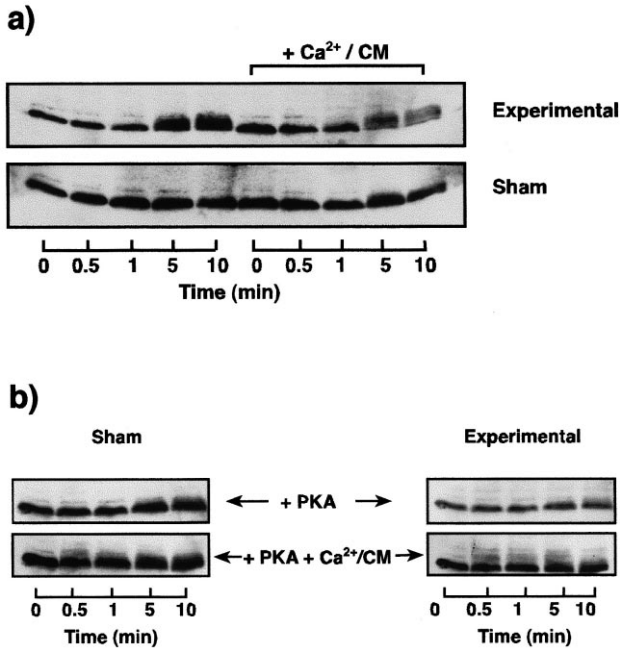

Fig. 3. Effect of added Ca^{2+} and calmodulin in the presence or absence of PKA pre-treatment on PLB phosphorylation. a: SR preparations were incubated at 30°C for increasing times in the presence of ATP and the presence or absence of added Ca^{2+}/CaM for the indicated times. b: Preparations were incubated as before but for increasing times in the presence of a sub-optimal level of PKA (0.2 μM). The effect of subsequent addition of Ca^{2+}/CaM was tested at increasing times of incubation as indicated. Reactions were terminated by addition of 4×Laemmli sample buffer and subjected to electrophoresis as described. PLB was detected using the A1 antibody as before. Results are from a single experiment, representative of three others.

rylated species of PLB as early as 30 s after addition of Ca^{2+} and CaM. Increased phosphorylation was evident in both sham and failure preparations but was more marked in the latter (Fig. 3b). Addition of PKA and resultant serine phosphorylation of PLB appears to aid further phosphorylation by a Ca^{2+}/CaM dependent kinase associated with the SR. This corresponds with the increased levels of phosphorylation seen in our previous studies examining intrinsic kinase activity associated with SR preparations from experimental hearts, particularly in freshly isolated left ventricular tissue that could exhibit up to 10 different phosphorylated forms of PLB. The fact that by stimulating serine phosphorylation above basal levels (although not maximally) we were able to increase CaM kinase activity suggests that there may be some degree of dependency in how the SR kinase pathways are regulated. Previous work by other groups has been contradictory in explanation of how these pathways interact. Some suggest the presence of dual control systems operating in an additive manner to regulate SR function [15] whereas others disagree with the idea of a coordinated dual control system and have demonstrated that sequential phosphorylation has to occur with threonine phosphorylation being dependent on prior serine phosphorylation [16]. This latter argument agrees with our findings in the present study, suggesting intrinsic SR CaM kinase activity is boosted by the initial activation of PKA.

The use of inhibitors directed against CaM kinase II is another way to highlight the involvement of this enzyme in PLB phosphorylation in these studies. We have previously used the inhibitor KN-62 [17] and found that even at maximal doses (up to 10 μM) there was no specific inhibition of rabbit CaM kinase. In the present study we used a specific peptide inhibitor (281–309) which contains both the calmodulin binding site (290–309) and the autophosphorylation site (Thr-286/7) of CaM kinase II. This inhibitor works by blocking Ca^{2+}/CaM activation and the enzyme active site. Fig. 4. shows that at 20 μM inhibitor, a maximal concentration still specific for CaM kinase, the upper phosphorylated band of PLB disappeared over time, indicating this inhibitor was preventing activation of CaM kinase II and subsequent phosphorylation of PLB in this rabbit SR preparation. It therefore seems likely that the upper phosphorylated bands of PLB are a result of CaM kinase II activation.

Further confirmation that the increase in phosphorylation in experimental preparations may be due to an SR associated

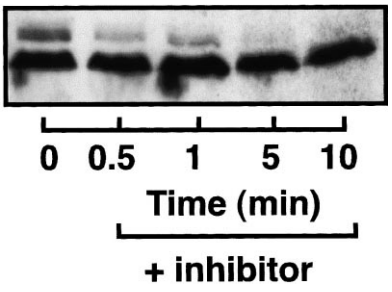

Fig. 4. Effect of CaM kinase II peptide inhibitor on intrinsic SR kinase activity. SR preparations were incubated for the indicated times as before in the presence of 2 mM ATP and the presence or absence of the peptide (281–301) at 20 μM. Reactions were terminated by the addition of 4×Laemmli sample buffer and subjected to electrophoresis as described. PLB was detected with the A1 antibody. Results are from a single experiment, representative of two others.

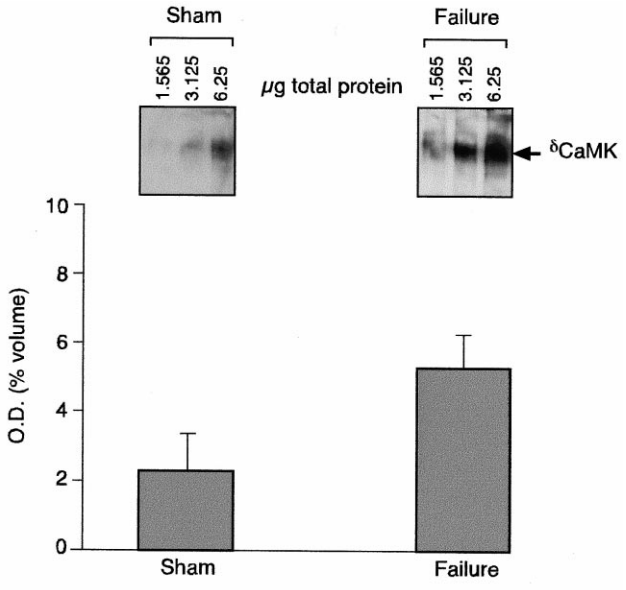

Fig. 5. Identification of δ CaM kinase by immunoblot analysis. Increasing protein loads for SR preparations from hearts of sham-operated and experimental animals were electrophoresed and probed with a specific rabbit anti-δ CaM kinase antibody. Immunoblots were developed and then subjected to densitometry analysis. Results are expressed as optical density units and represent the mean ± S.E.M. from a single typical experiment.

CaM kinase was obtained by specific strong positive staining of 56–58 kDa bands in SR preparations that had been prepared for electrophoresis (Fig. 5). This was observed using an anti-δ specific antibody that had been raised against the C-terminus of this subclass of CaM kinase [13]. These bands correspond to distinct isoforms of δ CaM kinase. Densitometry analysis on sham and experimental preparations using this antibody revealed increased expression of δ CaM kinase in experimental preparations by approximately 2.5-fold. This is a similar value to the increased activity of CaM kinase associated with these preparations observed in Fig. 2.

Additional immunoblotting experiments were performed on the same preparations probing for γ CaM kinase. Using the maximal concentration of this antibody recommended by the suppliers, we could detect no evidence of the γ subclass in either sham or experimental preparations (results not shown). Very recent work by another group has suggested increased transcript expression of δ CaM kinase II in failing human myocardium [18]. The present study strengthens this proposal and for the first time, not only shows the presence of the δ isoform in rabbit myocardium, but also presents evidence for increased expression of this protein in an animal model of heart failure.

Previous studies have suggested that δ CaM kinase is associated with cardiac SR in other species, however, as far as we are aware, there is only one indicating that the levels of this enzyme may be altered under pathophysiological conditions in the heart [18]. Evidence also exists however, for increased CaM kinase II activity in rat hippocampus after long-term ischaemia [19]. This is likely to be a different isoform. However, it highlights the possibility of up-regulation of CaM kinase in diseased tissue. Although we have focused on PLB phosphorylation by this enzyme, it is highly likely that other substrates for this enzyme exist within the SR. It is possible

that phosphorylation of PLB and other SR proteins that regulate Ca^{2+} uptake and release will contribute to increased myocardial relaxation. This would be particularly important in heart failure when these mechanisms are impaired. Studies by our group and others have suggested that the levels of PLB and SERCA 2 are decreased in heart failure [7,20,21] and we suggested that compensatory intracellular mechanisms may be activated under these conditions. One such mechanism may involve increased expression and/or activity of CaM kinase. Other studies in rat cardiac myocytes and SR preparations have suggested the existence of an SR kinase that is regulated by lumenal Ca^{2+} [22]. This 'state of filling' or SOF kinase phosphorylates PLB when Ca^{2+} is depleted in the SR, promoting refilling of the Ca^{2+} store. The identity of this kinase is unknown, however, the regulatory principle behind activation may be similar to the kinase in the present study i.e. under conditions where Ca^{2+} uptake is limited, kinase activation promotes PLB phosphorylation, which in turn promotes uptake by SERCA 2. As well as changes in kinase activities, it seems possible that there will also be a corresponding change in SR associated phosphatase levels. It has already been shown that protein phosphatase 1 (PP1) has to be inhibited to allow significant activation of CaM kinase [23]. PP1 dephosphorylates and deactivates CaM kinase and may well affect other SR related kinases. Already, drugs are being synthesised that specifically modulate certain signalling molecules that have shown to be altered during heart failure [24,25]. Without doubt, the present studies will result in a clearer understanding of how the functioning of the SR is altered in failure. They may also create more scope for specific therapeutic modulation at a cellular or sub-cellular level with advanced pharmacological or molecular tools.

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