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Ischemia induces phospholamban dephosphorylation via activation of calcineurin, PKC- α , and protein phosphatase 1, thereby inducing calcium overload in reperfusion

Kaori, Shintani-Ishida, Ken-ichi, Yoshida

Department of Forensic Medicine, Graduate School of Medicine, The University of Tokyo,
Tokyo, Japan

Address correspondence to: Kaori Shintani-Ishida, PhD. Hongo 7-3-1, Bunkyo-ku, Tokyo
113-0013, Japan. Tel: +81-3-5841-3367; Fax: +81-3-5841-3366; E-mail: kaori@m.u-
tokyo.ac.jp

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ABSTRACT

Cardiac sarcoplasmic reticulum (SR) Ca^{2+} ATPase (SERCA2a) promotes Ca^{2+} uptake in the SR. Dephosphorylated phospholamban (PLB) inhibits SERCA2a activity. We found a distinct dephosphorylation of PLB at Thr¹⁷ and Ser¹⁶ after 20-30 min of ischemia produced by coronary artery occlusion in rats. The aim of the study was to investigate how PLB is dephosphorylated in ischemia and to determine whether PLB dephosphorylation causes myocardial hypercontraction and calpain activation through Ca^{2+} overload in reperfusion. Protein inhibitor-1 (I-1) specifically inhibits protein phosphatase 1 (PP1), the predominant PLB phosphatase in heart. A Ca^{2+} -dependent phosphatase calcineurin may also induce PLB dephosphorylation. Ischemia for 30 min induced PKC- α translocation, resulting in inactivation of I-1 through PKC- α -dependent phosphorylation at Ser⁶⁷. The PP1 activation following I-1 inactivation was thought to induce PLB dephosphorylation in ischemia. Ischemia for 30 min activated calcineurin, and pre-treatment with a calcineurin inhibitor, cyclosporine A (CsA), inhibited PKC- α translocation, I-1 phosphorylation at Ser⁶⁷, and PLB dephosphorylation in ischemia. Reperfusion for 5 min following 30 min of ischemia induced spreading of contraction bands (CBs) and proteolysis of fodrin by calpain. Both CsA and an anti-PLB antibody that inhibits binding of PLB to SERCA2a reduced the CB area and fodrin

breakdown after reperfusion. These results reveal a novel pathway via which ischemia induces calcineurin-dependent activation of PKC- α , inactivation of I-1 through PKC- α -dependent phosphorylation at Ser⁶⁷, and PP1-dependent PLB dephosphorylation. The pathway contributes to the spreading of CBs and calpain activation through Ca²⁺ overload in early reperfusion.

Research highlights

Ischemia induces phospholamban (PLB) dephosphorylation via calcineurin activation

The activated calcineurin induces activation of PKC- α and protein phosphatase 1

Ischemia-induced PLB dephosphorylation contributes to calcium overload in reperfusion

1. Introduction

Sarcoplasmic reticulum (SR) Ca^{2+} ATPase (SERCA2a) uptakes Ca^{2+} into the SR, thereby regulating the cytosolic Ca^{2+} concentration and the rate of contraction and relaxation in the heart. Phospholamban (PLB) inhibits ventricular SERCA2a activity through binding to SERCA2a. When PLB is phosphorylated at Thr¹⁷ by calmodulin-dependent kinase II (CAMKII) and at Ser¹⁶ by protein kinase A (PKA), SERCA2a is activated upon release from its complex with PLB. Sustained SERCA2a activation through PLB phosphorylation underlies the enhanced cardiac contractility in the β -adrenergic response through enhanced SR Ca^{2+} loading [1].

In failing hearts, PLB is dephosphorylated and binds to SERCA2a, thereby depressing contractility through inhibition of the SR Ca^{2+} uptake [2,3,4]. Inhibition of PLB dephosphorylation by okadaic acid, a protein phosphatase inhibitor [5], inhibition of the PLB-SERCA2a interaction by PLB-targeted antibodies [6,7], or antisense ablation of PLB [8] improves cardiac contractility via enhanced SR Ca^{2+} loading facilitated by SERCA2a in the failing heart. These findings are consistent with the hypothesis that the SR acts as a Ca^{2+} sink for an excessive intracellular Ca^{2+} and that compromised SR uptake of Ca^{2+} may render cardiomyocytes sensitive to the intracellular Ca^{2+} overload.

In the heart, protein phosphatase 1 (PP1) is the predominant PLB phosphatase [5,9], and protein inhibitor 1 (I-1) is the main inhibitor of PP1 [10,11]. I-1 is activated by phosphorylation at Thr³⁵ (P-Thr³⁵) by PKA, with resultant inhibition of PP1 activity [12,13,14], and is inhibited by protein kinase C (PKC)-dependent phosphorylation at Ser⁶⁷ and/or Thr⁷⁵ [15,16,17]. PKC activation is accompanied by translocation between subcellular compartments. In ischemic perfused rat heart, we showed translocation of PKC- α and - ϵ from the cytosolic fraction to the particulate fraction [18].

Few reports support the involvement of a Ca²⁺-dependent phosphatase, calcineurin in PLB dephosphorylation. In the spontaneously hypertensive rat, a calcineurin inhibitor, cyclosporin A (CsA), normalizes blunted β -adrenergic responsiveness by prevention of PLB dephosphorylation and improvement of SR Ca²⁺ handling [19]. In dilated cardiomyopathic hearts, enhanced calcineurin activity mediates reductions in SERCA2a activity and SR Ca²⁺ uptake, and suggested that calcineurin directly dephosphorylates PLB at Thr¹⁷ [4]. Calcineurin is activated in acute myocardial ischemia [20].

There are few reports on the calcineurin-dependent PKC activation in hearts. In cardiomyocytes, calcineurin is necessary for stretch-induced PKC- ϵ translocation [21]. In transgenic mice overexpressing N-terminal fragment of K_v4.2 (K_v4.2N), calcineurin is activated and contributes to activation of PKC- α and - θ , as result from enhanced Ca²⁺ influx

[22]. In calcineurin overexpressing transgenic mice, calcineurin induces activation of PKC- α and θ , and is involved in hypertrophic response [23].

During the first minutes of post-ischemic reperfusion, cardiomyocytes undergo intercellular Ca^{2+} overload [24], resulting in propagation of contraction bands (CBs), a hallmark of Ca^{2+} -dependent myocardial hypercontraction [25], and in proteolysis of α -fodrin, a cytoskeletal protein by a Ca^{2+} -dependent protease, calpain [26]. The current study was based on our finding of the marked dephosphorylation of PLB at Thr¹⁷ and Ser¹⁶ after brief ischemia. We sought to find the link between PLB dephosphorylation during ischemia and Ca^{2+} overload during reperfusion.

In this study, we tested the hypothesis that brief ischemia induces calcineurin activation, PKC- α activation, PKC- α -dependent phosphorylation at Ser⁶⁷ of I-1 and inactivation of I-1, PP1 activation, and PLB dephosphorylation in an *in vivo* model of acute myocardial infarction. To address the role of these pathways in Ca^{2+} overload during early reperfusion, CBs and calpain-mediated fodrin proteolysis were examined after brief reperfusion.

2. Materials and methods

2.1. Animal model

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication 85-23, revised 1996) and was approved by the Institutional Animal Care and Use Committee of the University of Tokyo. The left anterior descending coronary artery (LAD) of male Sprague-Dawley rats aged 8 weeks was occluded. An ECG (lead II) was monitored throughout the experiment. Rats in which arrhythmic events (PVCs, short runs of VT, and occurrence of VF) occurred during the early period (5 to 10 min) of ischemia were used in subsequent analyses. After the experiment, 1% Evans blue (EB) dye was injected into the right atrium to demarcate the risk area in the heart. The heart was divided into risk (negative staining with EB) and non-risk (positive staining with EB) areas, and each specimen was stored. In further experiments, CsA (25 mg/ml/kg in saline, i.p. [27]) or Gö6976 (100 µg in 8 µl dimethylformamide, s.c. [28]) was injected 30 min before LAD occlusion, or FK506 (3.5 mg/0.1 ml/kg in dimethylformamide, i.v. bolus) was administered 15 min before LAD occlusion [20].

2.2. Sample preparation for Western blot analysis

For Western blotting, frozen heart tissue (0.1 g) was homogenized with 1ml of ice-cold STE buffer containing 320 mM sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 50 mM

NaF, 1 mM Na_3VO_4 and Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany). To examine PKC translocation, the homogenate was centrifuged at $10,000 \times g$ for 10 min, and the supernatant was further centrifuged at $100,000 \times g$ for 60 min. The $10,000 \times g$ pellet was suspended in ice-cold STE buffer in a final volume of 1 ml. The $100,000 \times g$ supernatant and $10,000 \times g$ pellet fractions are referred to as the cytosolic fraction and particulate fraction, respectively [18].

To enrich I-1 from heart tissues for Western blot detection, trichloroacetic acid (TCA) extraction was performed [13]. Briefly, frozen heart tissue (0.2 g) was homogenized with 2 ml of ice-cold 1.5% TCA containing 4 mM EDTA and centrifuged at $20,000 \times g$ for 30 min (unless otherwise specified). The supernatant was mixed with TCA at a final concentration of 19%, and the mixture was incubated at 4°C for 16 h and centrifuged. The pellet was resuspended in 500 nM Tris-HCl (pH 8.0), boiled for 10 min, and centrifuged. The supernatant was dialyzed for 16 h against distilled water at 4°C , centrifuged, and frozen at -80°C until further use.

Protein concentrations were determined by the Bradford method using a Coomassie Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA).

2.3. Western blot analysis

The homogenate (25 μg protein for PLB and fodrin 150 kDa fragment, 50 μg for mouse IgG) was subjected to SDS-PAGE on a 15% (PLB), 6.5% (fodrin) or 12.5% (mouse IgG) gel. For I-1, the TCA extract (5 μg protein) was subjected to SDS-PAGE on a 15% gel. To examine PKC translocation, the homogenate (5 μg protein), the cytosolic fraction (30 μg), and the particulate fraction (20 μg) were subjected to SDS-PAGE on 7.5% gels. Antibodies to P-Thr¹⁷ PLB, P-Ser¹⁶ PLB, SERCA2a, P-Thr³⁵ I-1, P-Ser⁶⁷ I-1, and PKC- θ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), anti-total PLB antibody was purchased from Calbiochem (Darmstadt, Germany), antibodies to PKC- α and - ϵ were obtained from BD Biosciences (Franklin Lakes, NJ), and anti-total α -fodrin antibody was purchased from Biohit (Helsinki, Finland). An antibody for detection of the fodrin 150 kDa fragment [29,30] was a generous gift from Dr. K Inomata (Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan). For chemiluminescence detection, peroxidase-conjugated anti-mouse, -rabbit, or -goat IgG was used (Promega, Madison, WI). The band densities were measured using CS analyzer 3 (Atto, Tokyo, Japan). The protein level of the target was determined from a standard curve constructed by plotting the band densities.

2.4. Measurement of calcineurin activity

Calcineurin activity was measured using a calcineurin activity assay kit (BioMol, Plymouth Meeting, PA) according to the instructions provided by the manufacturer. In brief, frozen tissues were homogenized in ice-cold lysis buffer and centrifuged at $10,000 \times g$ for 45 min. Phosphatase activity in the supernatant (0.5 mg protein/ml) was measured using RII Phosphopeptide as a calcineurin-specific substrate in the presence or absence of 10 mM EGTA. The phosphatase activity with EGTA treatment was subtracted from that that without EGTA treatment, and the subtracted phosphatase activity was regarded as calcineurin activity.

2.5. *In vivo* anti-PLB antibody injection

A mouse anti-PLB monoclonal antibody 2D12 (Abcam, Cambridge, UK) was injected into rat hearts using a hemagglutinating virus of Japan (HVJ)-envelope vector. The vector was provided by Ishihara Sangyo Kaisha (Osaka, Japan) and prepared as previously described [31]. HVJ-envelope vector (0.63 AU in 200 μ l saline) containing the antibody (12.5 μ g) was transfected into the heart at 4 sites (50 μ l at each site) 1 h before LAD occlusion. As a control, empty vector or vector containing mouse IgG (IgG2a monoclonal, Abcam) was

injected. To confirm induction of IgG in heart tissues, Western blots of the heart homogenate were probed with peroxidase-conjugated rat anti-mouse IgG with negligible cross-reactivity with rat serum protein (Jackson ImmunoResearch, West Grove, PA).

2.6. Immunofluorescence analysis

To examine the subcellular localization of the injected anti-PLB antibody, immunohistochemical staining for mouse IgG and SERCA2a was performed. Transverse sections of heart were immersed in 8% formaldehyde, embedded in paraffin, and sectioned at a thickness of 3 μm . The sections were deparaffinized and subjected to microwave treatment with citrate buffer for 5 min to expose antigen binding sites. The sections were then incubated overnight with goat anti-SERCA2a antibody (Santa Cruz Biotechnology) diluted 1:50, and then for 1 hour with Alexa Fluor 569 F(ab') fragment anti-goat IgG (as a secondary antibody for SERCA2a) and Alexa Fluor 488 anti-mouse IgG (to detection of injected anti-PLB antibody) (Invitrogen, Carlsbad, CA), each diluted 1:100. Fluorescence was observed with a confocal laser scanning microscope (Fluoview FV300, Olympus, Tokyo, Japan).

2.7. SR Ca²⁺ uptake assay

Cardiac SR vesicles were prepared according to the method of Vittone *et al.* [32]. The Ca²⁺ uptake capacity of the vesicles was measured as previously described [33]. Briefly, SR vesicles (0.2 mg/ml) were incubated with 2.5 μ M fluo-3 (Dojindo, Kumamoto, Japan) solution containing 0.15 mM potassium gluconate, 1 mM MgCl₂, 0.2 mM EGTA-calcium buffer (free [Ca²⁺] 0.3 μ M), 10 mM NaN₃, and 20 mM MOPS, pH 6.8. Upon addition of 0.5 mM ATP, the fluo-3 fluorescence is reduced by SR Ca²⁺ uptake. The fluorescence was monitored using a plate-reader (GENios, Tecan, Männedorf, Switzerland) with excitation at 485 nm and emission at 535 nm.

2.8. Measurement of the CB area

The CB area was measured 5 min after reperfusion following 30 min of ischemia, as described previously [25]. After staining of 3 μ m sections with phosphotungstic acid hematoxylin (PTAH), hypereosinophilic CB bands was measured. CB-positive cells were marked using digital image editing software Photoshop 6.0 (Adobe Systems, San Jose, CA). The risk area and the CB area were measured using Image-Pro Plus (Media Cybernetics, Bethesda, MD).

2.9. Statistical analysis

Group data are expressed as the means \pm SE. The data were analyzed using Dunnett test, Tukey-Kramer test, or Student t-test at a confidence level of 95%.

3. Results

3.1. Effects of calcineurin inhibitors on PLB dephosphorylation in ischemia

Western blots probed with antibody to total PLB showed a major pentameric band (30 kDa, PLB_p) and a minor monomeric band (6 kDa, PLB_m) (Fig. 1A). Western blots probed with specific antibodies demonstrated that PLB phosphorylated at Thr¹⁷ (P-Thr¹⁷ PLB) or Ser¹⁶ (P-Ser¹⁶ PLB) markedly decreased in the risk areas after 20-30 min of LAD occlusion, compared with the non-risk area (Fig. 1). Consistent with previous reports that phosphorylation of PLB promotes aggregation of PLB monomers into pentamers [34,35], monomeric PLB (PLB_m) increased in the risk areas after 20 and 30 min of LAD occlusion (total PLB in Fig. 1A), whereas total PLB level (PLB_p + PLB_m) was unaltered throughout ischemia (total PLB, in Fig. 1B). There was no significant change in the SERCA2a level throughout ischemia.

Ischemia for 30 min decreased P-Thr¹⁷ and P-Ser¹⁶ PLB levels to about 20% and 30% of the sham levels, respectively (Fig. 2). However, administration of a calcineurin inhibitor, CsA, before ischemia restored the P-Thr¹⁷ and P-Ser¹⁶ PLB levels in the risk area to about 70% and 95% of the sham levels, respectively, after 30 min of ischemia (Fig. 2A). In non-risk area, CsA had no effect on PLB phosphorylation (data not shown). A more specific calcineurin inhibitor, FK506, also abolished the post-ischemic dephosphorylation of PLB at Thr¹⁷ and Ser¹⁶ (Fig. 2B). Additionally, calcineurin activity increased in 30 min of ischemia, and the activation was inhibited by the CsA pre-treatment (Fig. 2C). These results suggest involvement of calcineurin in PLB dephosphorylation in ischemia.

3.2. Translocation of PKC- α in ischemia

After 30 min of ischemia, the PKC- α level decreased in the cytosolic fraction (Fig. 3B) and increased in the particulate fraction (Fig. 3C), but was unaltered in the homogenate (Fig. 3A). PKC- α is activated upon translocation in the risk area and pretreatment with CsA inhibited this translocation of PKC- α during ischemia (Figs. 3A-C). There was no PKC- α redistribution in non-risk area, irrespectively of CsA pre-treatment (data not shown). It has also been reported that calcineurin induces translocation of PKC- ϵ [21] and - θ [22,23].

However, we found that neither ischemia nor CsA had an effect on the distribution of PKC- ϵ or - θ (Fig. 3D).

Treatment with a synthetic PKC- α inhibitor, Gö6976, before LAD occlusion inhibited reduction of the levels of P-Thr¹⁷ and P-Ser¹⁶ PLB in 30 min of ischemia (Fig. 3E). These results indicate that calcineurin-dependent activation of PKC- α contributes to PLB dephosphorylation in ischemia.

3.3 Effects of ischemia and CsA administration on I-1 activity

Phosphorylation of I-1 at Ser⁶⁷ by PKC- α results in inactivation of I-1 [15], whereas phosphorylation at Thr³⁵ by PKA leads to activation of I-1 [12]. Ischemia for 30 min increased I-1 phosphorylation at Ser⁶⁷ to 2.5-fold (Fig. 4A) and decreased I-1 phosphorylation at Thr³⁵ to about 5% (Fig. 4B) compared with sham treatment. CsA pretreatment abolished I-1 phosphorylation at Ser⁶⁷ (Fig. 4A), but had no effect on dephosphorylation of I-1 at Thr³⁵ in ischemia (Fig. 4B). These results indicate that calcineurin-PKC- α -dependent phosphorylation of I-1 at Ser⁶⁷ contributes to PLB dephosphorylation in ischemia.

3.4. Relationship between PLB dephosphorylation and Ca²⁺ overload in reperfusion

PLB dephosphorylation and reduced SR Ca^{2+} uptake through SERCA2a inactivation in ischemia are likely to contribute to Ca^{2+} overload in early reperfusion. CB develops through Ca^{2+} overload in the first minutes of reperfusion [24] and we previously found that reperfusion following brief ischemia induces calpain-mediated proteolysis of fodrin (250 kDa) to the 150 kDa fragment [26]. After 5 min of reperfusion following 30 min of ischemia, CBs occupied about 20% of the risk area (empty+IR group in Fig. 6A, empty group in Fig. 6B). Reperfusion induced fodrin proteolysis with an increase in the 150 kDa fragment (saline groups in Figs. 7A and B).

The monoclonal anti-PLB antibody 2D12 was shown previously to block formation of the PLB complex with SERCA2a [7,36]. To examine whether PLB dephosphorylation in ischemia contributes to Ca^{2+} overload in reperfusion, we injected the risk area with the anti-PLB monoclonal antibody using the HVJ-envelope vector before ischemia and evaluated the CB area and fodrin degradation. Introduction of the antibody was confirmed by immunohistochemistry 1 h after the injection. Mouse IgG (injected anti-PLB antibody 2D12) immunofluorescence and SERCA2a immunofluorescence were co-distributed to the striations after the injection of the antibody (Fig. 5A), reflecting the presence of the antibody in the SR. Additionally, Western blot analysis showed the presence of the mouse IgG in the

risk area after anti-PLB antibody or IgG2a (as a control IgG) injection, but not in the non-risk area or in the risk area of the group injected with an empty vector (Fig. 5B).

Next, to examine whether the injected anti-PLB antibody blocks formation of the PLB-SERCA2a complex, we measured Ca^{2+} uptake in SR vesicles isolated from heart injected with anti-PLB antibody (2D12) or control IgG (IgG2a). After 30 min of ischemia, the amount of SR Ca^{2+} uptake in the risk area of IgG2a group decreased (70 % vs. sham) (Fig. 5C). Injection of 2D12 antibody blunted the decrease in the Ca^{2+} uptake (Fig. 5C).

After 5min of reperfusion following 30 min of ischemia, CBs occupied about 20 % of the risk area in hearts injected with empty vector (empty+IR group in Fig. 6A, empty group in Fig 6B). Injection of anti-PLB antibody decreased the CB area to about 4% of the risk area (2D12+IR group in Fig. 6A, 2D12 group in Fig. 6B), but injection of IgG2a had no effect on the CB area (IgG2a group in Fig. 6A, IgG2a group in Fig. 6B). There was no difference in the risk area between each group (Fig. 6B). In the sham-operated heart, injection of the anti-PLB antibody did not induce CBs (2D12+sham group in Fig. 6A). Dephosphorylation of PLB was not reduced after reperfusion for 5 min, compared with ischemia for 30 min (Figs. 6C and 1). Additionally, PLB dephosphorylation in risk area was not affected by the antibody injection (Fig. 6C).

Reperfusion for 5 min also induced fodrin proteolysis with generation of the 150 kDa

fragment, and this was blocked by anti-PLB antibody (Figs. 7A and B). Moreover, CsA pretreatment reduced the CB area (Fig. 6D) and fodrin degradation (Fig. 7) after reperfusion. These results support the hypothesis that calcineurin-mediated PLB dephosphorylation during ischemia contributes to Ca^{2+} overload-mediated injury in early reperfusion, which manifests as CB development and fodrin proteolysis.

4. Discussion/conclusion

The results of this study suggest the presence of a novel pathway involving calcineurin, PKC- α , I-1 and PP1 in dephosphorylation of PLB in ischemia (as summarized in Fig. 8). The pathway contributes to myocardial hypercontraction (contraction band, CB) and calpain activation due to Ca^{2+} overload in early reperfusion. CsA inhibited every step in this pathway: activation of PKC- α , phosphorylation of I-1 at Ser⁶⁷, and dephosphorylation of PLB at Ser¹⁶ and Thr¹⁷ during brief ischemia; and CB development and calpain activation during early reperfusion. FK506, a more specific calcineurin inhibitor, also inhibited PLB dephosphorylation at Ser¹⁶ and Thr¹⁷ after ischemia.

We showed for the first time that brief ischemia (20-30 min) induced marked dephosphorylation of PLB at Ser¹⁶ and Thr¹⁷. There are a few reports on PLB

dephosphorylation in ischemia-reperfusion with respect to hypertrophy or heart failure after myocardial infarction [11,37,38]. However, such marked PLB dephosphorylation has not been shown previously in an *in vivo* or *ex vivo* model of ischemia. This discovery may provide a breakthrough to a better understanding of the mechanism of formation of myocardial lesions and injury in acute ischemia-reperfusion.

Ischemia induced dephosphorylation of I-1 at Thr³⁵ and phosphorylation at Ser⁶⁷ (Fig. 4), either of which can inactivate I-1 [13,15]. CsA pre-treatment almost abolished dephosphorylation of PLB (Fig. 2) and phosphorylation of I-1 at Ser⁶⁷ (PKC- α site) (Fig. 4), but had no effect on dephosphorylation at Thr³⁵ (PKA site) in ischemic heart. Therefore, phosphorylation of I-1 at Ser⁶⁷ is likely to play a pivotal role in PLB dephosphorylation in ischemia. This study also showed a new pathway of regulation of PLB phosphorylation through PKC phosphorylation of I-1 downstream of calcineurin.

In neonatal rat cardiomyocytes, El-Armouche *et al.* demonstrated that calcineurin and PP2A constantly dephosphorylate I-1 at Thr³⁵ [39]. In this study, the ineffectiveness of CsA (Fig. 4) suggests that PP2A rather than calcineurin contributes to the dephosphorylation of I-1 at Thr³⁵ in ischemia. PKC- α can phosphorylate not only Ser⁶⁷ but also Thr⁷⁵ on I-1 [16], and the phosphorylation of either site induces inactivation of I-1 and promotion of PP1-dependent dephosphorylation of PLB at Ser¹⁶ [16,17]. The possible contribution of the

phosphorylation of I-1 at Thr⁷⁵ could not be neglected because of the lack of commercially available antibody. However, the nearly complete inhibition of the phosphorylation of I-1 at Ser⁶⁷ (Fig. 4) and PLB dephosphorylation (Fig. 2) by CsA supports that Ser⁶⁷ of I-1 is involved in PLB dephosphorylation in ischemia.

Ischemia induced PKC- α translocation to the particulate fraction *in vivo* (Fig. 3), as previously reported [18]. The PKC- α translocation was inhibited by CsA (Figs. 3A-C). Ischemia is well known to induce Ca²⁺ influx [40] and activate calcineurin [41]. In calcineurin overexpressing transgenic mice, calcineurin-dependent activation of PKC- α and - θ was demonstrated, but it remains unclear whether the activated calcineurin activates them directly or indirectly [23]. There is no report on the direct interaction of calcineurin and PKC- α . However, the activated calcineurin dephosphorylates L-type Ca²⁺-channels and enhances the Ca²⁺ influx [42], which can induce activation of a Ca²⁺-dependent PKC- α isoform [18]. The contribution of PKC- α to dephosphorylation of PLB at Ser¹⁶ and Thr¹⁷ in ischemia for 30 min was confirmed by the effect of Gö6976, a relatively specific PKC- α inhibitor (Fig. 3E). Involvement of other PKC isoforms (ϵ , θ) was negated by the lack of ischemia-induced translocation of PKC- ϵ and - θ and the lack of an effect of CsA on translocation of these two proteins in ischemia (Fig. 3D).

I-1 is a specific and prominent inhibitor of PP1 in the heart [10]. Previous reports indicate

PP1 activation in ischemia [5,43] and there are several studies showing dephosphorylation of PLB by PP1. In cardiomyocytes isolated from rats with chronic myocardial infarction, PP1 was suggested to induce PLB dephosphorylation based on the increase in PP1 activity and inhibition of PLB dephosphorylation by okadaic acid [5]. Okadaic acid is an inhibitor of PP1 and PP2A, but is difficult to use *in vivo*. Additionally, active I-1 expression augmented contractile function and recovery in *ex vivo* ischemia-reperfusion through SERCA2a activation via inhibition of PP1-mediated PLB dephosphorylation, although PLB dephosphorylation in ischemia was not shown in the study [36]. Collectively, these findings support a primary contribution of PP1 in the PLB dephosphorylation in ischemia.

Dephosphorylated PLB reduces SR Ca^{2+} uptake by binding to SERCA2a *in vitro* [44]. In a failing human heart, an increase in calcineurin expression is associated with dephosphorylation of PLB at Thr¹⁷ and reduced SERCA2a activity [4]. In spontaneously hypertensive rat, CsA normalizes the impaired contractile response to β -adrenergic stimulation through recovery of PLB phosphorylation [19]. Treatment of cardiomyocytes with anti-PLB monoclonal antibody enhances the rate of Ca^{2+} uptake and suppresses the ability of β -adrenoceptor stimulation to enhance SR Ca^{2+} handling [36]. In cardiomyopathic hamster, gene transfer of a PLB-targeted antibody improves Ca^{2+} handling and cardiac function [6]. Moreover, targeted antisense ablation of PLB rescues Ca^{2+} -overload-induced

contractile dysfunction in rats through SERCA2a activation [8]. However, the effect of anti-PLB antibody on reperfusion injury has not been examined in any experimental setting.

Reduced SR Ca^{2+} uptake caused by PLB dephosphorylation with impaired Ca^{2+} handling in ischemia is likely to induce reperfusion injury due to cytosolic Ca^{2+} overload during early reperfusion. Previously, we reported rapid spreading of CBs in 5 min of reperfusion following 30 min of ischemia in the same model [25]. We also found that rapid proteolysis of fodrin by calpain, a Ca^{2+} -dependent protease, causes contractile dysfunction in reperfusion following brief ischemia in perfused heart [26]. In this study, reperfusion for 5 min after 30 min of ischemia induced development of CBs and fodrin proteolysis (Figs. 6 and 7). We found that introduction of anti-PLB antibody and CsA administration greatly reduced the CB area (Fig. 6) and fodrin proteolysis (Fig. 7) in reperfusion. These results support the hypothesis that a pathway leading to the PLB dephosphorylation in ischemia causes Ca^{2+} overload in early reperfusion.

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Figure legends

Fig. 1. Temporal changes of P-Thr¹⁷, P-Ser¹⁶ and total PLB levels during ischemia. Panel A shows representative Western blots for P-Thr¹⁷, P-Ser¹⁶ and total PLB, SERCA2a and actin (as an internal control). Panel B shows the band densities of P-Thr¹⁷, P-Ser¹⁶ and total PLB relative to the mean of the sham group. Values are shown as means \pm SE (n=4). R, risk area; NR, non-risk area; PLB_p, pentameric PLB; PLB_m, monomeric PLB. **, p<0.01 vs. sham (Dunnett test).

Fig. 2. Effect of CsA (Panel A) or FK506 (Panel B) administration on PLB dephosphorylation and calcineurin activity (Panel C) in 30 min of ischemia. Values are shown as means \pm SE (n=6). DMF, dimethylformamide. *, p<0.05; **, p<0.01 (Tukey-Kramer test).

Fig. 3. Translocation of PKC isoforms in 30 min of ischemia. Panels A, B and C show PKC- α levels in whole (standard homogenate), cytosolic and particulate fractions, respectively, prepared from the risk areas of hearts subjected to 30 min of ischemia. Values are shown as means \pm SE (n=4). *, p<0.05; **, p<0.01 (Tukey-Kramer test). Panel D shows PKC- ϵ and - θ

levels in the cytosol and particulate fractions. Values are shown as means \pm SE (n=4). I, ischemia. There was no significant differences among the sham, I+saline and I+CsA groups in any fractions (Tukey-Kramer test). Panel E shows the effect of Gö6976 administration on PLB dephosphorylation in 30 min of ischemia. Values are shown as means \pm SE (n=4). DMF, dimethylformamide. *, p<0.05; n.s., no significant (Tukey-Kramer test).

Fig. 4. Effect of CsA administration on I-1 phosphorylation in 30 min of ischemia. Panels A and B show the relative levels of P-Ser⁶⁷ and P-Thr³⁵ I-1, respectively. Values are shown as means \pm SE (n=4). **, p<0.01 (Tukey-Kramer test).

Fig. 5. Injection of anti-PLB antibodies into the myocardium. Panel A shows representative images of mouse IgG or SERCA2a immunofluorescence in hearts 1 h after injection of empty vector or anti-PLB monoclonal antibody 2D12. Panel B shows a representative Western blot of mouse IgG in hearts injected with empty vector or vector containing 2D12 or IgG2a. IgG, purified mouse IgG was used in the Western blot analysis as a molecular weight control. Panel C shows Ca²⁺ uptake of SR vesicles prepared from hearts subjected to 30 min of ischemia following injection of 2D12 or IgG2a. Values are shown as mean \pm SE (n=6).

Fig. 6. Effect of anti-PLB antibody injection or CsA administration on the CB area in 5 min of reperfusion following 30 min of ischemia. Panel A shows the area of CB (marked in black) in transverse sections of hearts that underwent ischemia-reperfusion (IR) following injection with empty vector (empty+IR), 2D12 antibody (2D12+IR), or IgG2a (as IgG control, IgG2a+IR). “2D12+sham” refers to sham-operated hearts injected with 2D12 antibody. The borders of the risk and non-risk areas are indicated by dotted lines. Panel B shows the ratio (%) of the risk area (RA) to the left ventricular (LV) area and the ratio of the CB area (CA) to the RA. Values are shown as means \pm SE (n=6). **, p<0.01 vs. empty vector (Dunnett test). Panel C shows Western blots of P-Thr¹⁷ and P-Ser¹⁶ PLB in the hearts presented in panel A. R, risk area; NR, non-risk area; S, sham. Panel D shows the ratios (%) of the risk and CB areas. Values are shown as means \pm SE (n=6). **, p<0.01 vs. saline (Student t-test).

Fig. 7. Effect of anti-PLB antibody injection or CsA administration on calpain activation after 5 min of reperfusion following 30 min of ischemia. Panels A shows representative Western blots detected with anti-total fodrin antibody or anti-the fodrin 150 kDa fragment antibody. Panel B shows the relative band densities of the fodrin 150 kDa fragment to the intact fodrin (250 kDa). Values are shown as means \pm SE (n=4). R, risk area; NR, non-risk

area. **, $p < 0.01$ vs. the risk area in the saline group (Dunnett test).

Fig. 8. Proposed pathway of calcineurin-mediated PLB dephosphorylation during ischemia.

The steps at which CsA, FK506, Gö6976 and anti-PLB antibody show inhibitive effects are indicated by a, b, c and d, respectively.

Figure 1 (revised)

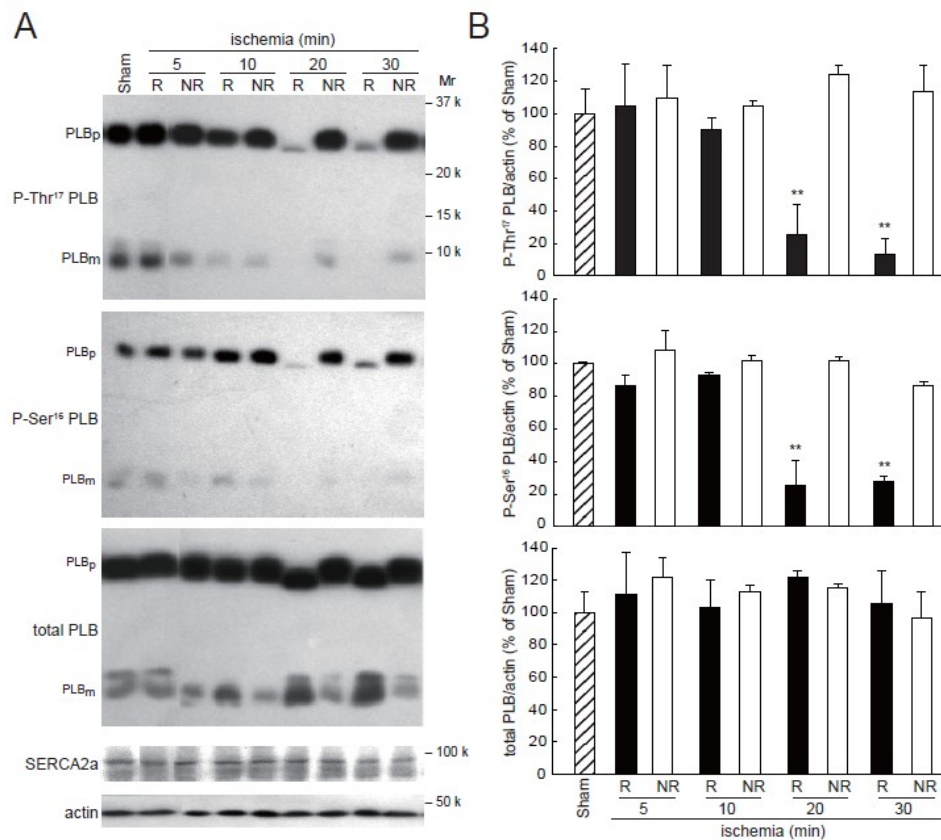


Figure 2

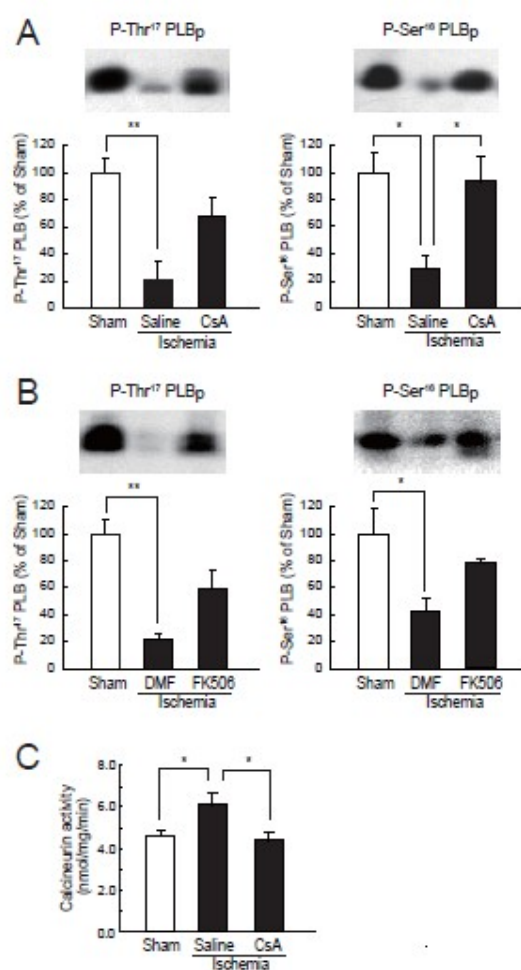


Fig. 2

Figure 3

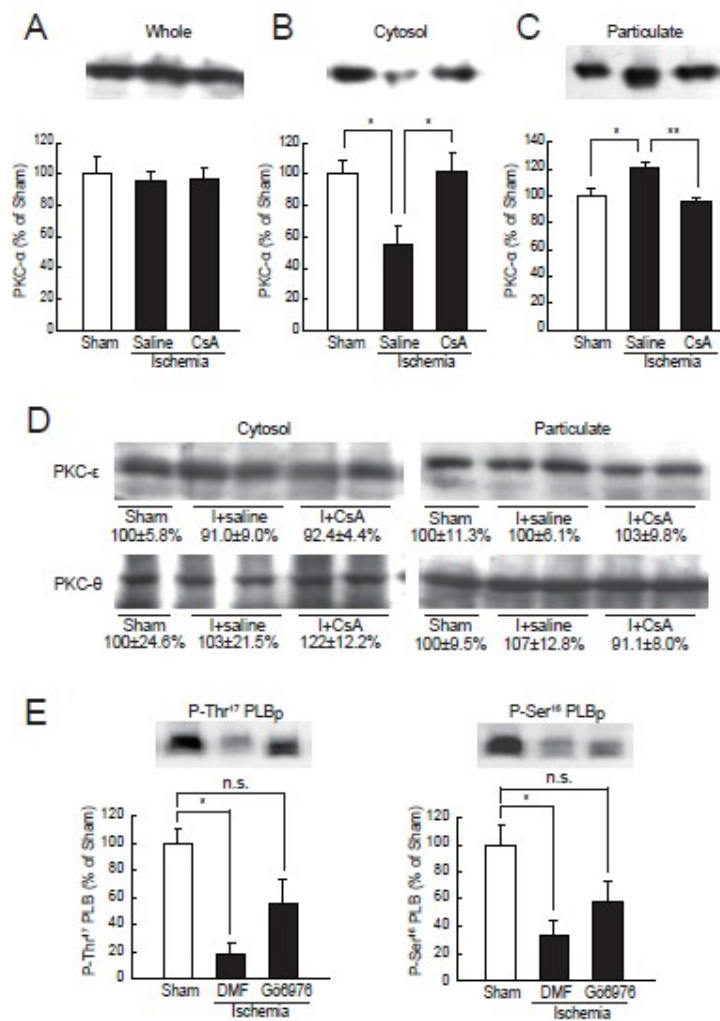


Fig. 3

Figure 4 (revised)

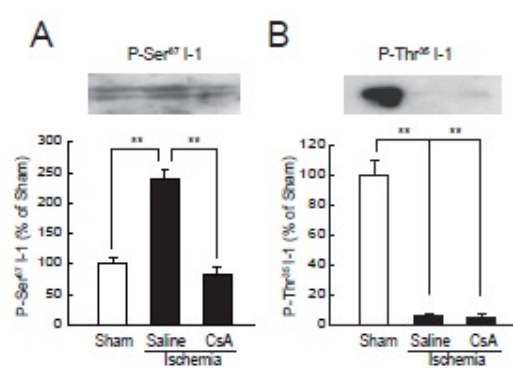


Fig. 4

Figure 5 (revised)

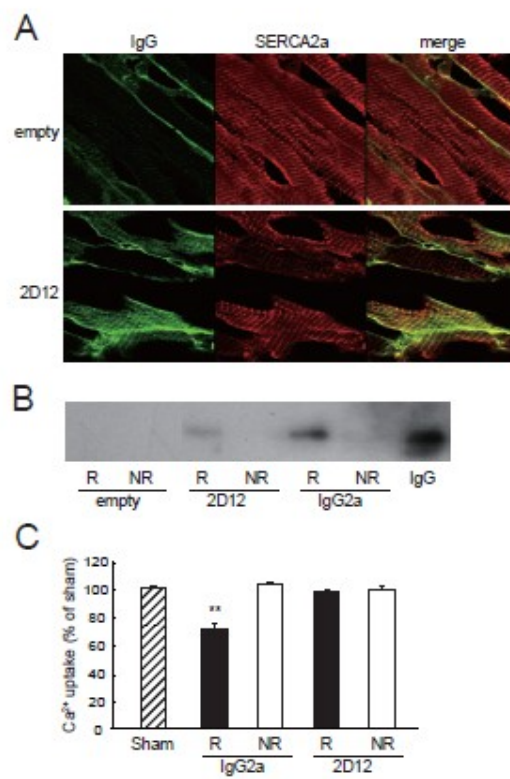


Fig. 5

Figure 6

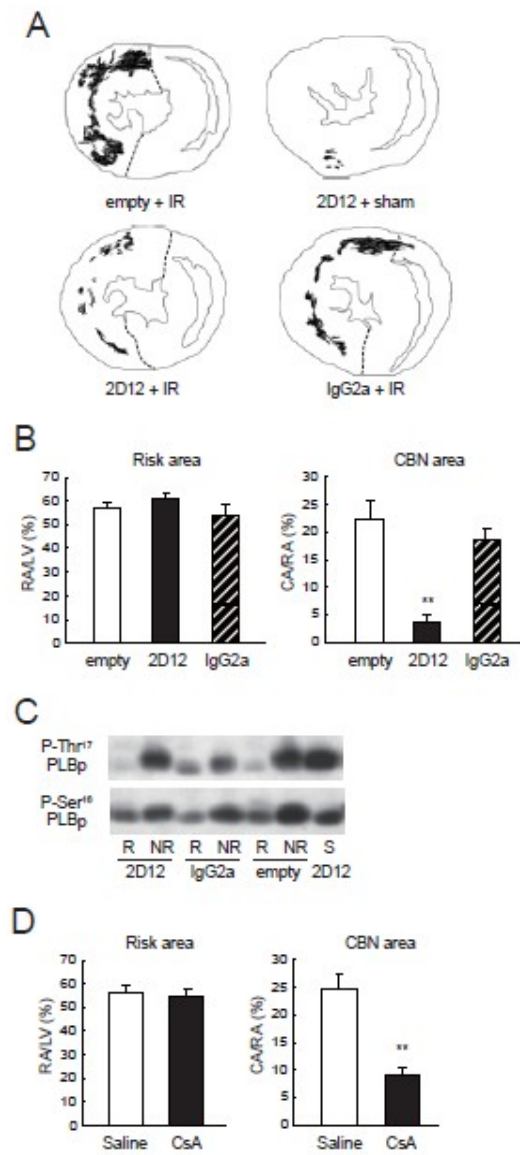


Fig. 6

Figure 7 (revised)

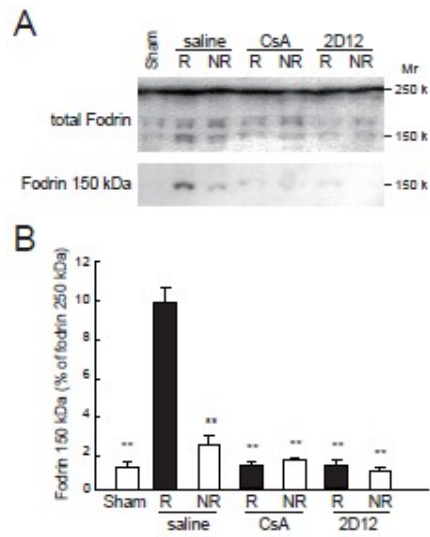


Fig. 7

Figure 8

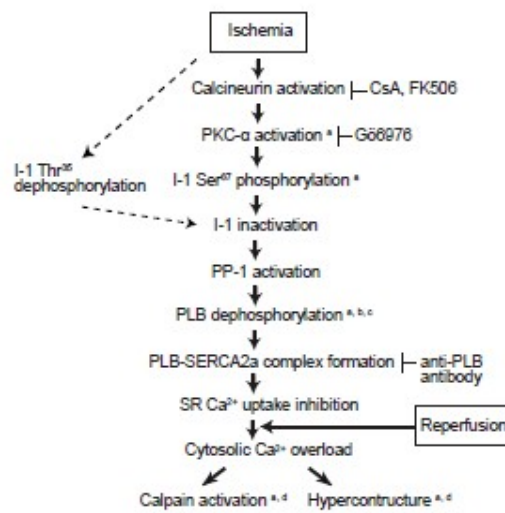


Fig. 8