Isoform-specific roles of protein phosphatase 1 catalytic subunits in sarcoplasmic reticulum-mediated Ca\(^{2+}\) cycling

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Aims
Protein phosphatase 1 (PP1) is the major isotype of serine/threonine phosphatase in cardiomyocytes, and its activity has been thought to be important for heart failure progression. The PP1 catalytic subunits consist of three distinct genes, PP1\(a\), PP1\(b/d\), and PP1\(g\). To date, the function of each PP1 isoform is not well characterized in cardiomyocytes. We sought to determine the functional contribution of each PP1 isoform to sarcoplasmic reticulum (SR)-mediated Ca\(^{2+}\) cycling in isolated adult rat cardiomyocytes.

Methods and results
Adenoviral vectors encoding short hairpin RNA for each PP1 isoform were transfected into isolated rat cardiomyocytes, and this was followed by analysis of cell shortening, Ca\(^{2+}\) transients, and the phosphorylation levels of Ca\(^{2+}\) regulatory proteins. Physical interactions between each PP1 isoform and SR Ca\(^{2+}\) regulatory proteins were characterized in isolated cardiomyocytes expressing green fluorescent protein (GFP)-tagged PP1 catalytic subunits, and also in canine junctional and longitudinal SR preparations. Successful PP1 isoform knockdown was achieved for each isoform without affecting the expression of the other isoforms. PP1\(b\) knockdown most significantly enhanced the Ca\(^{2+}\) transient and cell shortening by augmenting phospholamban (PLN) phosphorylation at baseline and with low-dose isoproterenol stimulation (10 nM). Interestingly, PP1\(b\) was preferentially associated with sarco-endoplasmic ATPase and PLN in GFP-PP1-transfected cardiomyocytes, as well as in canine longitudinal SR preparations.

Conclusion
These findings indicate that PP1\(b\) is the most significant PP1 isoform involved in regulating SR Ca\(^{2+}\) cycling in rat cardiomyocytes.

Keywords
Protein phosphatase 1 • Calcium cycling • Sarcoplasmic reticulum • RNAi • Rat cardiomyocytes

1. Introduction
The \(\beta\)-adrenergic system is the most powerful regulator of cardiac contraction and relaxation in the heart. Adrenergic stimulation leads to the activation of intracellular key kinases, such as A-kinase (PKA) and calcium/calmodulin-dependent kinase II (CAMKII), followed by target protein phosphorylations in the plasmalemma and sarcoplasmic reticulum (SR) and enhancement of intracellular Ca\(^{2+}\) cycling, resulting in positive inotropic and lusitropic actions of the heart.\(^1\) On the other hand, these phosphorylation events are reversibly regulated by protein phosphatases (PPs).\(^2,3\)

Cardiac PPs consist mainly of PP1, PP2A, and PP2B. We and others have shown that increased PP1 activity is closely associated with the progression of heart failure,\(^4\) and could be a potential therapeutic target.\(^4,5\) The PP1 catalytic subunit consists of three distinct genes: \(pp1ca\) (PP1\(a\)), \(pp1cb\) (PP1\(b/d\)), and \(pp1cc\) (PP1\(g\)),\(^6\) whose functions are not well characterized in cardiomyocytes. These three catalytic subunits have ~90% homology in the catalytic domain and 10% homology at the DNA level.\(^7\) Studies have indicated that PP1\(b\) is important for calcium handling during cardiac contractile activity.\(^8\)

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distinct sequences in the N and C termini, characteristics that are believed to be important for substrate and tissue specificities.9 Indeed, in non-cardiomyocyte experiments, it was reported that the three PP1 catalytic subunits were differentially distributed in HeLa cells,10,11 and each subunit governed a different cellular function.

Earlier microarray analysis using failing human hearts revealed that PP1α and PP1γ mRNA were downregulated, whereas that of PP1β was upregulated, in end-stage dilated cardiomyopathy.12 We therefore hypothesized that each PP1 catalytic subunit has a specific role in both the normal and diseased hearts.

The present study was designed to isolate PP1 isoform-specific function and to correlate this function with SR-mediated Ca^{2+} cycling in normal rat cardiomyocytes by using short hairpin RNA (shRNA)-mediated RNA interference technique. We found that PP1β is the most significantly involved isoform in regulating SR Ca^{2+} uptake in cultured cardiomyocytes.

2. Methods
An extended version of Methods is available in Supplementary material online.

2.1 Animals
All animal protocols were approved by the Yamaguchi University School of Medicine Animal Experiment Committee. The animals were treated according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Isolated adult rat left ventricular cardiomyocytes were prepared from normal male Wistar rats (6–8 weeks old, Japan SLC, Hamamatsu, Japan) as described previously.13 Male beagle dogs were anaesthetized by intravenous administration of thiopental (10 mg/kg) and ventilated, and the heart excised for further purification of cardiac SR.

2.2 Adenoviruses for shRNA vectors
Adenovirus (AdV) vectors encoding shRNA were designed to obtain a vector-based knockdown of PP1 isoforms. Briefly, self-annealing oligonucleotides for shRNA were designed using a web-based tool from Invitrogen (Carlsbad, CA, USA); target sense sequences (21 nt) of shRNA were inserted into the shRNA expression vector, pAdU6 (described in detail in Supplementary material online), followed by transfection of AdVs into HEK293 cells, as described previously.14 A scrambled PP1 shRNA sequence was used as a negative control vector (AdV.PP1-SCR). Among the AdV shRNA vectors created (two to three targets for each PP1 isoform shRNA), the following AdV.shRNA vectors were used for the PP1 isoform-specific knockdown experiment, as they showed significantly reduced protein levels without affecting the expression of other PP1 isoforms: AdV.PP1α-shRNA (position 382 of the PP1α mRNA) for PP1α RNAi, AdV.PP1β-shRNA (position 738 of the PP1β mRNA) for PP1β RNAi, and AdV.PP1γ-shRNA (position 829 of the PP1γ mRNA) for PP1γ RNAi.

2.3 Quantification of PP1 isoform expression
Cardiomyocytes were transfected with the AdVs at an MOI of 20, 200, and 500, and cultured for 24–72 h at 37°C in 5%CO₂/95%O₂ atmosphere. Cells were lysed with an ice-cold RIPA buffer containing 50 mM NaF, followed by quantitative immunoblotting using LAS-4000. The phosphorylation levels of phospholamban (PLN) at Ser16 and Thr17, that of ryanodine receptor (RyR) at Ser2808, and that of cardiac troponin I (Tnl) at Ser22 and 23 were normalized to the total protein levels. To normalize the phosphorylation level of PLN at Ser16 in the different immunoblots among baseline, 10 nM and 1 μM isoproterenol (ISO) stimulation, we estimated the coefficient, using quantitative image analysis (LAS-4000) (Supplementary material online, Figure S1). We used the coefficients to scale the phosphorylation level in the different immunoblots.

2.4 Immunoblot analysis of SR protein phosphorylation
For analysis of phosphorylation levels of SR proteins, cells were lysed in an ice-cold RIPA buffer containing 50 mM NaF, followed by quantitative immunoblotting using LAS-4000. The phosphorylation levels of phospholamban (PLN) at Ser16 and Thr17, that of ryanodine receptor (RyR) at Ser2808, and that of cardiac troponin I (Tnl) at Ser22 and 23 were normalized to the total protein levels. To normalize the phosphorylation level of PLN at Ser16 in the different immunoblots among baseline, 10 nM and 1 μM isoproterenol (ISO) stimulation, we estimated the coefficient, using quantitative image analysis (LAS-4000) (Supplementary material online, Figure S1). We used the coefficients to scale the phosphorylation level in the different immunoblots.

2.5 Measurement of cell shortening, Ca^{2+} transient, and SR Ca^{2+} load
For functional measurements, cardiomyocytes were transfected with the corresponding AdVs using an MOI of 500.

The %fraction of cell shortening (%CS) and the intracellular Ca^{2+} transient were simultaneously recorded at 60–72 h after AdV transfection while cells were field-stimulated with 1 Hz. This time period (60–72 h) was chosen because it contained the most significant shRNA knockdown effect without compromising cell damage after enzymatic isolation of cardiomyocytes. Measurements were done at baseline (~70 cells) and in the presence of 10 nM ISO (Sigma-Aldrich, St Louis, MO, USA). Intracellular Ca^{2+} changes were expressed as changes in ratio fluorescence measured at 340 and 380 nm.

The SR Ca^{2+} load was estimated as the peak fluorescence signal of fura-2 ratio after a 1 Hz stimulation train by rapidly switching the superfusion solution to one containing 20 mM caffeine, as described previously.15 The data obtained were further analyzed by IONOPTIX software (Milton, MA, USA).

2.6 Dephosphorylation assay of SR Ca^{2+} regulatory proteins
Cardiomyocytes were infected with either AdV.PP1-shRNA or AdV.PP1-SCR (control vector) at an MOI of 500, incubated at 37°C for 72 h, and then treated with 1 μM ISO for 1 h. Thereafter, cells were washed twice with ISO-free culture medium and harvested at 0, 5, 10, 20, 40, and 60 min, followed by analysis of the PLN phosphorylation levels (at Ser16 and Thr17) and RyR2 (at Ser2808).

2.7 AdV expression of EGFP-tagged PP1 isoform and pulldown of SR Ca^{2+} regulatory proteins
To characterize the physical interaction between each PP1 isoform and PLN/RyR in the SR, we created an AdV encoding an EGFP-tag for each PP1 isoform with a single amino acid mutation (D94/95N), and transfected each AdV into cardiomyocytes (details are provided in Supplementary material online).16 Forty-eight hours after transfection, the cells were lysed with a solution containing 50 mM NaCl, 50 mM Tris (pH 7.4), 2 mM MgCl₂, and 0.5% Triton X-100. EGFP-tagged PP1 protein complexes were purified with a μMACS green fluorescent protein (GFP)-tagged protein isolation kit (Miltenyi Biotec, Germany). The
physical associations between EGFP-PP1 mutants and SR Ca\(^{2+}\) regulatory proteins were further analysed.

2.8 Immunoprecipitation of PP1 and Ca\(^{2+}\) regulatory proteins
Cardiac microsomes were solubilized by the lysis solution. First, 150 µg of crude SR was pre-cleared with protein G sepharose (Amersham) for 30 min, and incubated with primary antibodies at 4°C overnight followed by precipitation with protein G sepharose for 1 h. Immunoblot analyses were performed to determine the physical interaction between PP1 isoform and SR proteins, such as PLN, sarco-endoplasmic ATPase 2a (SERCA2a), and RyR.

2.9 Isolation of cardiac microsomes and purification of junctional and longitudinal SRs
Cardiac microsomes were prepared as described previously.\(^{17}\) A centrifugation technique using two consecutive sucrose density gradients (the protocol is described in detail in Supplementary material online) in combination with Mg\(^{2+}\)-ATP-dependent SR Ca\(^{2+}\) overloading was employed to isolate two different fractions of the SR—the junctional and longitudinal SR—without significant contamination by other organelles, as was previously confirmed by electron microscopy.\(^{18}\)

2.10 Antibodies
The following antibodies were obtained from commercially available sources: antibodies for PP1\(\alpha\) (BD Biosciences, San Jose, CA, USA), PP1\(\beta\) (ab16369, ab53315), PP1\(\gamma\) (ab26175, ab16387), and PLN (ab2865, clone 2D12) (Abcam, Cambridge, UK); phosphorylated PLN at Ser16, PLN, PKA catalytic subunit, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chemicon International), phosphorylated PLN at Thr17, and phosphorylated-RyR2 at Ser2808 (Badrilla, Leeds, UK); RyR2 and \(\alpha\)-actinin (Sigma-Aldrich), SERCA2a (clone N-19; Santa Cruz Biotechnology, Santa Cruz, CA, USA), cardiac TnI (clone 19C7), and phospho-TnI at Ser22 and 23 (clone S66) (Genetex, San Antonio, TX, USA).

2.11 Statistical analysis
Comparisons between the two groups were performed by Student’s t-test. Comparisons between repeated measurements were done with ANOVA, followed by the post hoc test (the Student–Newman–Keuls method was used to compare the two groups when appropriate). A value of \(P<0.05\) was considered statistically significant. Data are expressed as the mean \(\pm\) SEM.

3. Results
3.1 Efficiency of PP1 isoform-specific shRNA
AdVs encoding shRNA vectors for PP1\(\alpha\), PP1\(\beta/\delta\), and PP1\(\gamma\) were transfected into adult rat cardiomyocytes, and representative knockdown efficiency was shown at 0, 48, and 72 h after transfection on the protein expression level (Figure 1A). Expression levels in each PP1 isoform was most significantly reduced at 72 h. All PP1 isoform-specific PP1-shRNA vectors showed significant reduction (Figure 1B: PP1\(\alpha\) 59.1 \(\pm\) 3.0% vs. control; Figure 1C: PP1\(\beta\) 64.1 \(\pm\) 2.1% vs. control; Figure 1D: PP1\(\gamma\) 77.7 \(\pm\) 3.4% vs. control) at 72 h, although

Figure 1 Effective and isoform-specific knockdown of PP1\(\alpha\), \(\beta\), and \(\gamma\) using AdV-mediated shRNA vectors. (A) Representative immunoblot of each PP1 isoform after AdV PP1-shRNA transfection. Protein samples of cell lysates at 0, 48, and 72 h were electrophoresed and analysed by immunoblotting with each PP1-specific antibody. Note that each PP1-shRNA reduced the expression level of the corresponding PP1 isoform. GAPDH was used as a loading control. (B–D) Summarized data of PP1-shRNA-mediated knockdown of each PP1 catalytic subunit at 72 h after transfection (Panel B: PP1\(\alpha\), Panel C: PP1\(\beta\), Panel D: PP1\(\gamma\)). *\(P<0.05\) compared with the control (AdV.PP1-SCR-transfected) group \((n = 4\) in each group).
the differences in shRNA efficiency among the three PP1 isoforms were not statistically significant. A significant shRNA effect in the mRNA level was also confirmed at 48 h after transfection (see Supplementary material online, Figure S2). In addition, those shRNA vectors did not induce a significant interferon response (see Supplementary material online, Figure S3), supporting the PP1-shRNA-mediated specific gene knockdown. None of the PP1 isoform shRNA had any effect on cell survival or the subcellular SR and Z-band structure, as assessed by immunofluorescence of RyR2, PLN, and α-actinin (Supplementary material online, Figure S4A). None of the PP1 knockdown significantly altered PP1 activity in cardiomyocyte homogenates (Supplementary material online, Figure S4B), whereas AdV inhibitor-2 expression significantly depressed PP1 activity. Thus, the successful knockdown of each PP1 isoform was accomplished without affecting the expression of the other PP1 isoforms. Interestingly, none of the PP1 knockdowns affected the whole-cell PP1 activity in isolated rat cardiomyocytes, suggesting that there is a compensatory mechanism to maintain a constant level of whole-cell PP1 activity.

3.2 Effects of PP1-shRNA on phosphorylation levels of SR proteins

Although none of the individual PP1 knockdown effects altered whole-cell PP1 activity, the basal phosphorylation levels of PLN at Ser16 were significantly increased by PP1β-shRNA in an MOI-dependent manner (Figure 2A, p16-PLN, arrows, and Figure 2B). PP1α-shRNA also slightly increased PLN phosphorylation at Ser16.

**Figure 2** Changes in the phosphorylation levels of key SR proteins after PP1 isoform-specific knockdown. (A) Representative immunoblotting of key phosphoproteins in cardiomyocytes 72 h after transfection at baseline (BL). PLN, phospho-specific PLN at Ser16, phospho-specific PLN at Thr17, RyR, phospho-specific RyR at Ser2808, cardiac TnI, and phospho-specific TnI at Ser22 and 23. GAPDH was used as a loading control. Immunoblotting of PLN and phospho-Ser16 PLN is shown with low-dose ISO (10 nM) and high-dose ISO (1 μM) stimulation. The arrows indicate increased phosphorylation levels of PLN at baseline (the single arrows in the PP1α- and PP1β-shRNA groups) and with low-dose ISO stimulation (the double arrows in the PP1α- and PP1β-shRNA groups). (B–D) Quantification of phosphorylation levels at Ser16-PLN at baseline (Panel B: BL) and with low-dose (Panel C: ISO 10 nM) and high-dose ISO stimulation (Panel D: ISO 1 μM) (n = 5 in each experiment). *P < 0.05 compared with the control group (AdV.PP1-SCR).
Figure 3  PP1β knockdown most effectively augmented the amplitude and decay speed of the Ca$^{2+}$ transient and cell shortening. (A) Representative tracing of %CS and the Ca$^{2+}$ transient at 72 h after transfection with AdVP1-shRNA vectors. Cardiomyocytes were stimulated by a 1 Hz electrical pulse at baseline (black line) and at 10 nM ISO stimulation (red line). SCR represents a control vector that contains a random scramble sequence (SCR) of the PP1-shRNA. (B–G) Summary of cell-shortening and Ca$^{2+}$ transient parameters (%CS, Panel B), the maximum and minimum values of the first derivative of cell length, i.e. $+dL/dt$ (Panel C) and $-dL/dt$ (Panel D), the amplitude (Panel E), and the decay time constant (Panel F) of the Ca$^{2+}$ transient. Panel G represents the baseline Ca$^{2+}$ level at the diastolic phase. *$P < 0.05$ compared with the control group; #$P < 0.05$ compared with the PP1α-shRNA group; $^{†}P < 0.05$ compared with the PP1γ-shRNA group.
(small arrows), but the effect of PP1α-shRNA on PLN phosphorylation did not reach statistical significance (Figure 2A and B). There were no changes in the expression levels in PLN, RyR, SERCA2a, and the PKA catalytic subunits, in phosphorylation levels of PLN at Thr17, cardiac TnI at Ser22 and 23, or RyR2 at Ser2808 (Figure 2A and see Supplementary material online, Figure S5) by AdV-mediated PP1-shRNA. There were no changes in the expression levels of PP1 endogenous inhibitors, namely inhibitor-1 and inhibitor-2 in PP1-shRNA-treated cardiomyocytes (Supplementary material online, Figure S6).

The increased phosphorylation level of PLN at Ser16 was further enhanced by PP1β- and PP1α-shRNA with low-dose ISO (10 nM) stimulation (Figure 2A middle, double arrows, and Figure 2C). However, such enhancement effects became indistinguishable at high-dose ISO (1 μM) (Figure 2A bottom and D). These data indicate that PP1β-shRNA, and partially PP1α-shRNA, can alter phosphorylation balance of PLN at Ser16 without β-adrenergic stimulation in cultured cardiomyocyte.

### 3.3 PP1β-shRNA most effectively enhanced cell shortening and the Ca2+ transient

Among these PP1 knockdowns, the PP1β knockdown most effectively augmented the amplitude of Ca2+ transient, %CS, positive (+$\Delta L/dt$) and negative (−$\Delta L/dt$) first derivatives of the cell length both at baseline and with low-dose ISO stimulation, as shown in Figure 3A–G. Although the PP1α and PP1γ knockdowns also slightly enhanced %CS and the Ca2+ transient, it was in both cases to a lesser extent than with PP1β (Figure 3B–G). To rule out the Ca2+ buffering effects that the dye Fura-2 might have on the cell capacity to respond to stimulation, we also performed experiments in unloaded cells transfected with PP1β-shRNA. Under these experimental conditions, we observed similar enhancement of %CS (data not shown).

Furthermore, the SR Ca2+ load was significantly enhanced in PP1β-shRNA-treated cardiomyocytes but not in other PP1 isoform-shRNAs (Figure 4A and B), supporting the notion that

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**Figure 4** Estimation of SR Ca2+ loads and dephosphorylation kinetics in AdV.PP1-shRNA-transfected cardiomyocytes. (A) Representative time courses of Ca2+ transients before and after the addition of caffeine in AdV.PP1-shRNA-transfected cardiomyocytes. The caffeine-induced increase reflecting the SR Ca2+ load was measured after a 1 Hz stimulation train by rapidly switching the superfusing solution to the one containing 20 mM caffeine. (B) Summarized data of estimated SR Ca2+ load in AdV.PP1-shRNA-transfected cardiomyocytes. *P, 0.05 vs. control group (AdV.PP1-SCR-transfected group). The numbers in each group are indicated in the columns. (C) Representative time course of phosphorylation levels of PLN at Ser16 and RyR at Ser2808 in PP1β-shRNA. The kinetics of dephosphorylation after ISO 1 μM stimulation were analysed by the time course of phosphorylation level with phospho-specific antibodies (PLN at Ser16, and RyR at Ser2808). (D and E) Summarized data of the time course of phosphorylation levels of PLN at Ser16 (D) and RyR at Ser2808 (E) after ISO stimulation in PP1β-shRNA and control (Ctl, AdV.PP1-SCR-transfected) group. n = 4 in each group; *P < 0.05 compared with the control group.
PP1β-shRNA most significantly enhanced SR Ca\(^{2+}\) uptake in cultured cardiomyocytes.

### 3.4 Effect of PP1β-shRNA dephosphorylation kinetics

As the PP1β knockdown most significantly enhanced SR Ca\(^{2+}\) handling and increased PLN phosphorylation, we also assessed the kinetics of dephosphorylation in PLN and RyR, key proteins in SR Ca\(^{2+}\) cycling, in PP1β-knockdown cardiomyocytes. As shown in Figure 4C, incubation with 1 μM ISO for 1 h largely phosphorylated PLN at Ser16 and RyR2 at Ser 2808. Thereafter, dephosphorylation levels were determined at each time point after ISO removal. In PP1β-shRNA cardiomyocytes, the time constant of dephosphorylation of PLN at Ser16 was slower than that of the control AdV.PP1-SCR-transfected cardiomyocytes (Figure 4D). The time courses of dephosphorylation of RyR at 2808 appeared to be unaffected by PP1β knockdown (Figure 4E). These data indicate that the kinetics of PLN dephosphorylation at Ser16 are also affected by PP1β-shRNA.

According to these findings, we further hypothesized that PP1β was more selectively involved with SERCA2a and PLN than other PP1 isoforms in the longitudinal SR of cardiomyocytes.

### 3.5 SERCA and PLN preferentially interact with PP1β

To quantitatively compare the avidity of the PP1 isoforms for SERCA2a and PLN, AdVs expressing N-terminally EGFP-fused PP1α, PP1β, and PP1γ with a single amino acid mutation (D94/95N; a catalytically inactive mutant) in their catalytic domains were transfected into cardiomyocytes, followed by purification with GFP antibody-binding microbeads and assessment of the physical interaction between PP1β and SERCA/PLN. As demonstrated in Figure 5A, EGFP-tagged PP1α, PP1β, and PP1γ were exclusively detected by each PP1 isoform antibody, indicating the specificity of the antibodies. Interestingly, SERCA and PLN were most abundantly associated with EGFP-PP1β (Figure 5B). The higher avidity of PP1β for PLN in terms of the endogenous expression levels was also confirmed by using canine microsome (crude SR) preparations. PP1β and PLN were co-immunoprecipitated in 0.5% Triton X-100-solubilized canine SR preparations (Figure 5C) but the PP1α and PP1γ antibodies were not, suggesting that endogenous PP1 preferentially interacts with PLN in the SR. In addition, RyR was co-immunoprecipitated with PP1β and PP1γ, but not with PP1α, suggesting a differential physical interaction between PP1 catalytic subunits and both PLN and RyR in the SR.

### 3.6 Three PP1 isoforms are differentially distributed in the junctional and longitudinal SR

To further elucidate PP1 isoform-specific regulation of SR Ca\(^{2+}\) cycling, intrinsic PP1 isoform distributions were further characterized in the canine SR, which were separated into plasmalemma, junctional SR, and longitudinal SR fractions. As we needed a large amount of heart tissue (∼120 g) for the SR subfractionation procedure, we used canine hearts instead of rat hearts for this analysis. In the crude SR, all PP1 isoforms were equally detected (Supplementary material online, Figures S7 and S8) by their specific antibodies. In the subfractionated SR (Figure 6A), PP1α was dominantly detected in the plasmalemma-rich fractions (fractions 1–3, as characterized by the enrichment of β-dystroglycan (β-DG) in Figure 6B). PP1β exhibited ubiquitous distribution throughout all of the SR fractions (fractions 1–6), and exhibited comparatively higher expression in the longitudinal SR fraction (Figure 6A, indicated by the arrow in the lane of fraction 6). PP1γ was highly enriched in the junctional SR (Figure 6B, indicated by the double arrow in the lane of fraction 5). The contamination by mitochondria and myofibrillar fraction was almost negligible, as only a very faint band was detected in fractions 1–6 (Figure 6B).

These data indicate that the junctional SR contains a greater amount of PP1γ and that the longitudinal SR contains a greater amount of PP1β.

### 4. Discussion

In the present study, we successfully achieved PP1 isoform-specific knockdown in cultured cardiomyocytes by using AdV PP1-shRNA vectors, and found that PP1β knockdown most significantly enhanced cell shortening and the Ca\(^{2+}\) transient among the three PP1 isoforms. Phosphorylation changes in PLN at Ser16, which were most significantly augmented by PP1β knockdown, at least partly explain the enhancement effect on cell shortening and the Ca\(^{2+}\) transient. In addition, we found that PP1β displayed higher expression in the longitudinal SR, and formed a molecular complex with PLN and SERCA2a, supporting the notion that PP1β is the most significantly involved
Figure 6 Differential distribution of PP1 isoforms in the junctional and longitudinal SR. (A) Scheme of two consecutive applications of the discontinuous sucrose density gradient centrifuge method. First centrifugation was done without Ca\(^{2+}\) and Mg\(^{2+}\), yielding two major fractions, namely the light microsome (light micro) and heavy microsome (heavy micro). Each microsome fraction was Ca\(^{2+}\)-overloaded by incubation with Ca\(^{2+}\) 10 mM, Mg\(^{2+}\) 10 mM, and ATP 10 mM at 37°C for 10 min, and then subjected to a second round of the discontinuous sucrose density gradient. (B) Immunoblot of SR-associated proteins in the subfractionated canine SR fractions. The contents of PLN, SERCA2a, and RyR2 are shown by immunoblots of the subfractionated SRs, namely the plasmalemmal (fractions 1–3), junctional SR (fractions 4–5), and longitudinal SR (fraction 6). PP1α, PP1β, PP1γ, and PP2A distributions are shown in each fraction by their specific antibodies. GRP78, cytochrome oxidase subunit 4 (COX4), myosin heavy chain (MHC), and β-DG were used as marker proteins of the endoplasmic reticulum, mitochondria, the myofibrillar fraction, and the plasmalemma fraction. (C) Schematic diagram of the SR in cardiomyocytes. The SR can be separated into two fractions: the junctional SR and longitudinal SR. The junctional SR consists of Ca\(^{2+}\)-releasing channel subunits, including RyR2 and its accessory proteins. The longitudinal SR consists of Ca\(^{2+}\) uptake components, including SERCA2a and PLN. According to the findings in the present study, the three PP1 isoforms are differentially distributed throughout the junctional and longitudinal SR. PP1β most effectively controls SR Ca\(^{2+}\) cycling and cell shortening by associating with PLN in the longitudinal SR.
isoform in regulating SR Ca\(^{2+}\) uptake. To our knowledge, this is the first demonstration of isoform-specific PP1 regulation of SR-mediated Ca\(^{2+}\) cycling in cardiomyocytes.

The three PP1 isoforms have been shown to play distinct roles related to dephosphorylation targets in a variety of cell types. For example, PP1\(\alpha\) has been shown to be most important for cell proliferation, and PP1\(\beta\) has been shown to be important for glycogen metabolism and myosin phosphorylation, whereas PP1\(\gamma\), including two alternative splice variants of PP1\(\gamma\)1 and PP1\(\gamma\)2, has been shown to play a specific role in spermatogenesis, apoptosis against oxidative stress in vascular smooth muscle, and neuronal function in a Parkinson disease model. In the heart, a previous study revealed that the PP1\(\alpha\) transcript appeared to be the highest among the three isoforms relative to the ribosomal RNA, followed by PP1\(\gamma\) and then PP1\(\beta\). As we were unable to measure any isoform-specific PP1 activity or quantify isoform-specific subcellular distribution by immunohistochemistry due to a lack of the appropriate antibodies, we chose shRNA-mediated gene knockdown to compare isoform-specific PP1 function on SR-mediated Ca\(^{2+}\) cycling. Adv-shRNA successfully reduced the genetic expression of each PP1 (59–78% of control). In particular, PP1\(\alpha\) and PP1\(\beta\) were almost equally suppressed by shRNA knockdown at the protein expression level (59.1 ± 3.0 and 64.1 ± 2.1% vs. control, respectively). Nevertheless, PP1\(\beta\)-shRNA significantly augmented the %CS and Ca\(^{2+}\) transient at baseline and with low-dose ISO, compared with PP1\(\alpha\)-shRNA, strengthening the idea that PP1\(\beta\) has a higher impact than PP1\(\alpha\) on SR Ca\(^{2+}\) cycling. Although the suppression of PP1\(\gamma\) tended to be slightly higher than that of PP1\(\alpha\) and that of PP1\(\beta\) at the protein level, its knockdown effect on Ca\(^{2+}\) cycling and cell shortening was lower, suggesting a minor role in SR-mediated Ca\(^{2+}\) cycling in cardiomyocytes.

PP1\(\alpha\) also significantly enhanced Ca\(^{2+}\) cycling and cell shortening at baseline and with low-dose ISO stimulation. One favoured explanation is that PP1\(\alpha\) was slightly but significantly detected in the longitudinal and junctional SR, and has been reported to be the most abundant PP1 isoform among the three isoforms in cardiomyocytes. It is conceivable that PP1\(\alpha\) function on SR Ca\(^{2+}\) cycling is not exclusively governed by PP1\(\beta\) but in part regulated by PP1\(\alpha\).

The whole-cell PP1 activity was not altered in any of PP1\(\alpha\)-shRNA-mediated knockdown. As PP1 catalytic subunits do not freely exist in the cell but are known to associate with a variety of regulatory subunits, the compensatory mechanism may be involved in the maintenance of the whole cellular PP1 activity constant. As previously reported from in vitro and in vivo experiments, certain parts of the PP1 isoform function can be compensated by other isoforms, supporting the fact that the whole-cell PP1 activity was kept constant in each PP1 isoform-specific knockdown. In addition, a lack of an effect of PP1\(\alpha\)-shRNA on the phosphorylation target, including RyR and Tnl, also supports the fact that the whole-cell PP1 activity was not altered by PP1\(\alpha\)-shRNA. Nevertheless, PP1\(\beta\)-shRNA effectively augmented PLN phosphorylation at Ser16 at baseline and with low-dose ISO stimulation, suggesting that the subcellular microenvironment in the vicinity of PLN was significantly affected by PP1\(\beta\)-shRNA and partially by PP1\(\alpha\)-shRNA.

On the other hand, PLN phosphorylation was not affected at high-dose ISO stimulation in PP1\(\alpha\)-shRNA-treated cardiomyocytes. It is conceivable that the augmentation effect of PLN phosphorylation by PP1\(\beta\)-shRNA was not appreciable in the condition of maximally phosphorylated PLN at Ser16 with high-dose ISO stimulation. In addition, the dephosphorylation kinetics of PLN phosphorylation were significantly altered by PP1\(\beta\)-shRNA. These data suggest that PP1\(\beta\) not only determines the static levels of phosphorylation but also affects the dynamics of phosphorylation/dephosphorylation of PLN at Ser16 in cardiomyocytes. As PP1\(\beta\) expression has been shown to gradually increase during the progressive time course of cardiac dysfunction in cardiomyopathic hamsters, the expression levels of PP1\(\beta\) may have a significant impact on SR Ca\(^{2+}\) cycling.

We found that there is a differential PP1 isoform-specific distribution in the longitudinal and junctional SR in cardiomyocytes. Figure 6). In particular, PP1\(\beta\) was comparatively enriched in the longitudinal SR, and formed a molecular complex with PLN and SERCA2a, supporting the notion that PP1\(\beta\) is mostly present in the longitudinal SR. These data may explain, in part, why PP1\(\beta\) knockdown exhibited the highest impact on cell shortening and the Ca\(^{2+}\) transient in isolated rat cardiomyocytes. Whether the physical interaction between PLN and PP1\(\beta\) is direct or indirect remains to be investigated. In this regard, the PP1 catalytic subunit is known to be anchored by a specific adapter protein in the corresponding subcellular component. One such possible partner for PP1\(\beta\) is RGL2 (also known as Gm), a PP1 glycogen-targeting subunit, rich in the SR, that has been reported to be preferentially associated with PP1\(\beta\) isoforms in skeletal muscles. Together, these data suggest that PP1\(\beta\) and, in part, PP1\(\alpha\) regulate local PP1 activity in close proximity of SERCA/PLN via PP1 regulatory proteins without changing whole-cell PP1 activity in cardiomyocytes, as proposed in Figure 6C.

Regarding the high junctional SR-specific distribution of PP1\(\gamma\), we could not determine a significant functional consequence on either Ca\(^{2+}\) cycling or RyR phosphorylation by the PP1 isoform knockdown experiments. It is conceivable that RyR phosphorylation is thus regulated by multiple PPs. Indeed, the recent characterization of RyR phosphorylation by site-specific antibodies revealed that Ser2808 is controlled by a combination of PP1 and PP2A, whereas Ser2815 is preferentially controlled by PP2A, suggesting that a single PP1 isoform knockdown may have only a minor impact on RyR phosphorylation.

In conclusion, we have demonstrated that PP1\(\beta\) is the most significantly involved PP1 isoform in regulating PLN phosphorylation at Ser16 in the longitudinal SR, thereby controlling SR-mediated Ca\(^{2+}\) cycling in cardiomyocytes. Because PP1\(\beta\) expression is significantly increased in the failing heart, modulation of its activity may be a potential therapeutic target. Whether PP1\(\beta\) inhibition is beneficial or detrimental to cardiac dysfunction needs to be investigated in future in vivo experimental settings.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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