

than Gq-coupled receptors. For example, in colorectal cancer cells, epidermal growth factor (EGF) increased expression of cyclooxygenase-2 via STIM1 and Orail [49]. In pulmonary arterial smooth muscle cells, platelet-derived growth factor (PDGF) activated SOCE via Akt signaling [50]. In addition, both EGF [51] and PDGF [52] can increase the activity of ERK signaling in melanoma. It will be interesting to investigate the functional relationship among tyrosine kinase-type receptors, SOCE and the ERK signaling in melanoma cells.

In conclusion, our results indicate that inhibition of SOCE suppressed proliferation and cell migration/metastasis of melanoma cells, most probably via ERK signaling. We propose that SOCE is a potential target for treatment of melanoma, irrespective of whether or not Braf mutation is present.

## Supporting Information

**Figure S1 STIM1-knockdown did not promote apoptosis.** APC Annexin V and 7-AAD staining assays demonstrated that apoptosis of STIM1-knockdown and control shRNA-transduced C8161 cells was not different. These data demonstrate that apoptosis did not contribute to the STIM1-knockdown-induced inhibition of cell proliferation. N.S. not significant, n = 4. (TIF)

**Video S1 Melanoma cell migration was inhibited by STIM1- or by Orail-knockdown.** Cellular movement was recorded by time-lapse video microscopy recording. Video S1,

control shRNA, video S2, STIM1 shRNA, video S3, Orail shRNA. (WMV)

**Video S2 Melanoma cell migration was inhibited by STIM1- or by Orail-knockdown.** Cellular movement was recorded by time-lapse video microscopy recording. Video S1, control shRNA, video S2, STIM1 shRNA, video S3, Orail shRNA. (WMV)

**Video S3 Melanoma cell migration was inhibited by STIM1- or by Orail-knockdown.** Cellular movement was recorded by time-lapse video microscopy recording. Video S1, control shRNA, video S2, STIM1 shRNA, video S3, Orail shRNA. (WMV)

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## Author Contributions

Conceived and designed the experiments: MU YI KI. Performed the experiments: MU EB LX XF KO AM. Analyzed the data: MU EB SF MSD XF MI. Contributed reagents/materials/analysis tools: SF MD LX UY TF SC JG. Wrote the paper: MU EB SFD LX MI YI KI.

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# Protection of Cardiomyocytes from the Hypoxia-Mediated Injury by a Peptide Targeting the Activator of G-Protein Signaling 8

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## Abstract

Signaling via heterotrimeric G-protein is involved in the development of human diseases including ischemia-reperfusion injury of the heart. We previously identified an ischemia-inducible G-protein activator, activator of G-protein signaling 8 (AGS8), which regulates G $\beta\gamma$  signaling and plays a key role in the hypoxia-induced apoptosis of cardiomyocytes. Here, we attempted to intervene in the AGS8-G $\beta\gamma$  signaling process and protect cardiomyocytes from hypoxia-induced apoptosis with a peptide that disrupted the AGS8-G $\beta\gamma$  interaction. Synthesized AGS8-peptides, with amino acid sequences based on those of the G $\beta\gamma$ -binding domain of AGS8, successfully inhibited the association of AGS8 with G $\beta\gamma$ . The AGS8-peptide effectively blocked hypoxia-induced apoptosis of cardiomyocytes, as determined by DNA end-labeling and an increase in cleaved caspase-3. AGS8-peptide also inhibited the change in localization/permeability of channel protein connexin 43, which was mediated by AGS8-G $\beta\gamma$  under hypoxia. Small compounds that inhibit a wide range of G $\beta\gamma$  signals caused deleterious effects in cardiomyocytes. In contrast, AGS8-peptide did not cause cell damage under normoxia, suggesting an advantage inherent in targeted disruption of the AGS8-G $\beta\gamma$  signaling pathway. These data indicate a pivotal role for the interaction of AGS8 with G $\beta\gamma$  in hypoxia-induced apoptosis of cardiomyocytes, and suggest that targeted disruption of the AGS8-G $\beta\gamma$  signal provides a novel approach for protecting the myocardium against ischemic injury.

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## Introduction

Signaling mediated by heterotrimeric G-protein plays important roles in signal integration in cells. Heterotrimeric G-proteins are activated by G-protein-coupled receptors (GPCRs) at the cell surface in response to extra stimuli. The activation of G-protein signaling is associated with nucleotide exchange on the G $\alpha$  subunits leading to a conformational change in G $\alpha\beta\gamma$  and subsequent transduction of signals to various effector molecules [1]. However, a novel class of regulatory proteins that directly activate heterotrimeric G protein without receptor activation has been identified [2–4]. Such molecules are expected to provide alternative signaling via heterotrimeric G-protein and regulate signal adaptation during pathophysiologic stress [5].

The importance of accessory proteins for heterotrimeric G-protein has been reported in human diseases and animal models [5]. For example, activator of G-protein signaling 1 (AGS1) is a direct activator of the G $\alpha$  subunit and involved in the secretion of atrial natriuretic factor in heart failure [6,7]. Further, regulators of G-protein signaling (RGSs), the group of proteins that inhibit G-protein signaling by accelerating the GTPase activity of the G $\alpha$  subunit, are involved in various cardiovascular diseases, such as

hypertension, cardiac hypertrophy, and hypoxia-mediated injury [8–10].

We have been focusing on identification of accessory proteins of heterotrimeric G-proteins induced in cardiovascular diseases and have found novel activators of G-protein signaling from the hypertrophied heart and during repetitive transient ischemia [11,12]. Thus, we identified TFE3 (AGS11), an AGS protein that selectively forms a complex with the G $\alpha$ 16 subunit and is upregulated in the hypertrophied hearts of mice [11]. TFE3 translocates G $\alpha$ 16 to the nucleus, which leads to the induction of claudin-14, a component of the membrane in cardiomyocytes. This suggests that the novel form of transcriptional regulation counteracts pressure overload.

Activator of G-protein signaling 8 (AGS8) is a G $\beta\gamma$  signal regulator isolated from a cDNA library of rat hearts subjected to repetitive transient ischemia [12]. In response to hypoxia, AGS8 is up-regulated in the myocardium and cultured adult cardiomyocytes. AGS8 interacts directly with G $\beta\gamma$  and promotes G $\beta\gamma$  signaling in cells [12]. Suppression of AGS8 inhibits hypoxia-induced apoptosis of cardiomyocytes, suggesting AGS8 is required for hypoxia-mediated cell death [13]. AGS8 complexes with

connexin 43 (CX43) to form a transmembrane channel for multiple small molecules, including calcium, adenosine, ATP, and reactive oxygen species [14–16]. AGS8 regulates phosphorylation of CX43 in a G $\beta\gamma$ -dependent manner and influences hypoxia-mediated internalization of cell-surface CX43 [13]. Therefore, the AGS8-G $\beta\gamma$  complex plays a critical role under hypoxic conditions, making the cellular environment more sensitive to hypoxic stress by influencing the permeability of molecules passing through CX43.

Ischemic injury of the heart is associated with activation of multiple signal cascades initiating intracellular ionic and chemical changes that lead to the death of cardiomyocytes [17,18]. A previous study indicated that AGS8-G $\beta\gamma$  is involved in the programs leading to cell death, and the formation of the AGS8-G $\beta\gamma$  complex appears to be a critical step triggering the apoptotic process [13]. If a tool to manipulate the AGS8-G $\beta\gamma$  interaction in cells were available, it might be a promising approach for protection of cardiomyocytes against hypoxia-mediated injury.

Here, we report the identification of the G $\beta\gamma$ -interface of AGS8 and a peptide (AGS8-peptide) designed to correspond to the domain of interaction between G $\beta\gamma$  and AGS8 that protects cardiomyocytes against hypoxia-induced apoptosis. The observations indicate the importance of the AGS8-G $\beta\gamma$  complex in hypoxia-mediated apoptosis of cardiomyocytes as well as the potential value of targeted disruption of the AGS8-G $\beta\gamma$  signal for protecting the myocardium against ischemic injury.

## Experimental Procedures

### Materials

Anti G $\beta$  antibody was obtained from Santa Cruz Biotechnology. Anti CX43 antibody and IGEPAL CA-630 was obtained from Sigma. Gallein was purchased from Calbiochem. Cleaved caspase-3 antibody was obtained from Cell Signaling Technology. Recombinant G $\beta_1\gamma_2$  was obtained from Calbiochem-Merck Millipore. MTT (3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Dojindo Molecular Laboratories (Kumamoto, Japan).

### Synthesis of Peptide

The peptides were synthesized and purified by Invitrogen and peptide identity was verified by matrix-assisted laser desorption ionization mass spectrometry. The peptides were dissolved in aliquots (10 mM) and immediately frozen at  $-70^{\circ}\text{C}$ .

### Generation of GST-fusion Protein, Protein Interaction Assays, and Immunoblotting

The segments of AGS8 (DQ256268) were amplified by PCR and fused in frame to GST in the pGEX-6T vector (Amersham Biotech). Each GST-partial-AGS8 fusion proteins were expressed in bacteria (*Escherichia coli* BL21, Amersham Biotech) and purified on a glutathione affinity matrix. The GST fusion protein was eluted from the resin, and glutathione was removed by desalting to allow a solution-phase interaction assay [19]. Protein interaction assays and immunoblotting were performed as described previously [19,20].

### Preparation of Cardiomyocytes and Delivery of Peptide to Cells

Cardiomyocytes were prepared from the hearts of 1-3-day-old Wistar rats as described previously [13]. The neonates were deeply anesthetized with pentobarbital sodium (100 mg/kg) and decapitated for cardiac tissue harvesting. The ventricular cardiomyo-

cytes were then enzymatically dissociated and seeded at  $1.0 \times 10^5$  cell in 24 mm or  $4.0 \times 10^5$  cell in 35 mm plates. Prepared cardiomyocytes were cultured in DMEM/F12 including Insulin-Transferrin-Selenite (ITS), 100 units/ml penicillin, 100 mg/ml streptomycin, and 10 mM glutamine, in 5% CO $_2$  at 37°C. In some experiments, cardiomyocytes were incubated in a hypoxic incubator (MODEL9200, Wakenyaku, Kyoto, Japan) equilibrated to 1% O $_2$ , 5% CO $_2$ , and 94% N $_2$  at 37°C. The cells were subjected to two different hypoxic challenges. In the first protocol, cardiomyocytes were exposed 3 times to 30 min of hypoxia with intermittent 30-min periods of normoxia to capture the early events caused by hypoxia in the living cells before they died. Particularly, internalization of cell-surface connexin 43 and changes in the permeability of connexin were analyzed. In the second protocol, cardiomyocytes were exposed to 1% O $_2$  for 6 h followed by 12 h of normoxia to induce hypoxia-mediated apoptosis. At the end of the second protocol, apoptotic cell death was analyzed. In some experiments, approximately 24 h after preparation, peptides were delivered to cardiomyocytes by using PLUSin (Polyplus, NY, USA) according to the manufacturer's instructions. Briefly,  $\sim 1.0$   $\mu\text{g}$  peptide was incubated with 2.0  $\mu\text{l}$  of PLUSin in 100  $\mu\text{l}$  supplied buffer for 15 min and then added the mixture to each dishes. This preparation typically provided 0.5 to 1  $\mu\text{M}$  of peptide in the culture medium. The amount of peptide and the incubation time for peptide-delivery were optimized by analyzing incorporation of fluorescein isothiocyanate (FITC)-conjugated peptide into cardiomyocytes. In the condition used in this study, the chemical reagent for peptide delivery did not influence the number of living cells analyzed by trypan blue stain within 4 h treatment (0.75  $\mu\text{M}$  peptide;  $102.0 \pm 6.7\%$ , 1.5  $\mu\text{M}$  peptide;  $90.5 \pm 5.0\%$  versus no treatment control, not statistically significant,  $n = 4$ ) [21].

### Immunocytochemistry

Immunostaining of cultured cardiomyocytes was performed as described previously [13]. Briefly, cells were seeded on 24 $\times$ 24 mm polylysine-coated coverslips. Cells were fixed with 4% paraformaldehyde for 15 min and then incubated with 0.2% Triton X-100 for 5 min. After 1 h incubation of 5% normal donkey serum, cells were incubated with primary antibodies for 18 h at 4°C. Following 1 h incubation of secondary antibody (goat anti-mouse AlexaFluor 488 or goat anti-rabbit AlexaFluor 594, highly cross-absorbed, Molecular Probes), cells were incubated with 1  $\mu\text{g}/\text{ml}$  4',6'-diamidino-2-phenylindole, dihydrochloride (DAPI) (Molecular Probes) in PBS for 5 min. Slides were then mounted with glass coverslips with ProLong Gold antifade reagent (Invitrogen). Images were analyzed by deconvolution microscopy (TE2000-E, Nikon, Tokyo, Japan). Obtained images were deconvoluted using NIS-Elements 3.0 software (Nikon) with a "no neighbors" deconvolution algorithm. All images were obtained from approximately the middle plane of the cells.

### Dye Uptake Study

Uptake of CX43 permeable fluorescence dye Lucifer Yellow (LY) (Molecular Probes) was performed as described previously [13]. Briefly, cardiomyocytes were incubated with 1 mM Lucifer Yellow (LY) (Molecular Probes) for 30 min. The fluorescence of LY was determined by fluorescence microscopy (B-3A filter, TE2000-E, NIKON, Tokyo, Japan) following removal of incorporated LY and rinse with PBS. The signal intensity was quantified in 10 randomly selected fields (10 $\times$ 20) using NIS-Elements 3.0 software (NIKON, Tokyo, Japan). The non-specific binding of LY was determined in the presence of a connexin hemichannel blocker, 50 mM of Lanthanum (Sigma-Aldrich).

### In situ Assay for Apoptosis Detection

In situ labeling of fragmented DNA in cardiac myocytes was detected by TACS2 TdT-Blue Label In Situ Apoptosis Detection Kit (Travigen, Inc., Gaithersburg, MD), that detects DNA breaks in genomic DNA by enzymatic incorporation of biotinylated nucleotides followed by the binding of streptavidin-peroxidase conjugates, according to the manufacture's instructions. Briefly, myocytes were fixed with 3.7% formaldehyde in phosphate buffered saline (PBS) for 10 min and with 70% ethanol for 5 min and then incubated in proteinase K (0.02 mg/ml) at room temperature for 5 min. The cells were incubated with 2% hydrogen peroxide for 5 min and washed with labeling buffer consisting of 50 mM Tris (pH 7.5), 5 mM MgCl<sub>2</sub>, 60 mM 2-mercaptoethanesulfonic acid, and 0.05% BSA, followed by 60 min of incubation at 37°C in labeling buffer containing 150 mM dATP, 150 mM dGTP, 150 mM dTTP, 5 mM biotinylated dCTP, and 40 U/ml of the Klenow fragment of DNA polymerase I. Untreated myocytes incubated with or without 2 mg/ml DNase in the labeling buffer were used as positive or negative controls, respectively. The incorporated biotinylated dCTP was then detected with streptavidin-peroxidase conjugate and revealed in 0.5 mg/ml diaminobenzidine for 10 min. Nuclear brown staining was viewed under a light microscope.

### MTT Assay

Cardiomyocytes in 24-well culture plates were incubated in PBS (pH 7.4) containing 0.5 mg/ml MTT at 37°C. After for 2 h incubation, the PBS was removed and 100 µl of dimethyl sulfoxide (DMSO) was added to each well to solubilize dark blue formazan products. Absorbance of the colored solution was determined at 595 nm [22].

### Miscellaneous Procedures and Statistical Analysis

Immunoblotting and data analysis were performed as described previously [12,13]. The luminescence images captured with an image analyzer (LAS-3000, Fujifilm, Tokyo, Japan) were quantified using Image Gauge 3.4 (Fujifilm). Data are expressed as mean ± S.E.M. from independent experiments as described in the figure legends. Statistical analyses were performed using the unpaired *t* test, F-test, one-way ANOVA followed by Tukey's multiple comparison post-hoc test. All statistical analyses were performed with Prism 4 (GraphPad Software, USA).

### Ethics Statement

Animal study was approved by the Institutional Animal Care and Use Committees of Yokohama City University.

## Results

### The C-terminal Region of AGS8 was Required to Activate Gβγ Signaling in Cells

To determine the region of interaction between Gβγ and AGS8, we divided the rat AGS8 (DQ256268) sequence into 6 segments and synthesized the segments as glutathione *S*-transferase (GST)-fusion proteins (Fig. 1A). Each of GST-AGS8 peptides was subjected to a pull-down assay to examine its interaction with purified Gβγ (Fig. 1B). The C-terminus of AGS8 (AGS8-C) successfully pulled down Gβγ, as documented in the previous manuscript [12]. Another segment, AGS8-254, also pulled down Gβγ to a lesser extent than AGS8-C, suggesting that a potential second Gβγ-interacting domain existed in this region. However, the remaining segments failed to pull down Gβγ.

Next, the bioactivity of each of the AGS8 regions was investigated by evaluating the activation the G-protein signaling pathway in *Saccharomyces cerevisiae* [12,23]. This yeast strain lacked the pheromone receptor, but expressed mammalian Gαs in place of the yeast Gα subunit and provided a read-out of growth upon activation of the G-protein-regulated pheromone signaling pathway [23]. The peptide corresponding to each AGS8 domain was subcloned in a galactose-inducible vector and introduced into the yeast strain [24]. The bioactivity in the G-protein pathway was examined by evaluating the galactose-dependent growth of the transformed yeast. Although each segment of AGS8 was expressed in the yeast cells, AGS8-C was the only segment able to activate G-protein signaling (Fig. 1C). Thus, we focused on AGS8-C and explored the Gβγ-interaction site in this domain.

### The FN3 Domain was Important for the Interaction of AGS8 with Gβγ

The sequence of AGS8-C (A<sup>1359</sup> to W<sup>1730</sup> of rat ABB82299) was further divided into smaller fragments that were synthesized as GST-fusion proteins in bacteria. Two fusion proteins of GST-AGS8-C, namely, AGS8-C1 (A<sup>1359</sup>–H<sup>1493</sup>) and AGS8-C2 (A<sup>1494</sup>–T<sup>1585</sup>), were successfully synthesized and migrated as expected on SDS-PAGE. While AGS8-C1 did not pull down purified Gβγ, AGS8-C2, which represented the FN3 domain, did pull down Gβγ, indicating the importance of the FN3 domain for the interaction of AGS8 with Gβγ (Fig. 1D).

### AGS8-peptides Blocked the Interaction of AGS8C with Gβγ

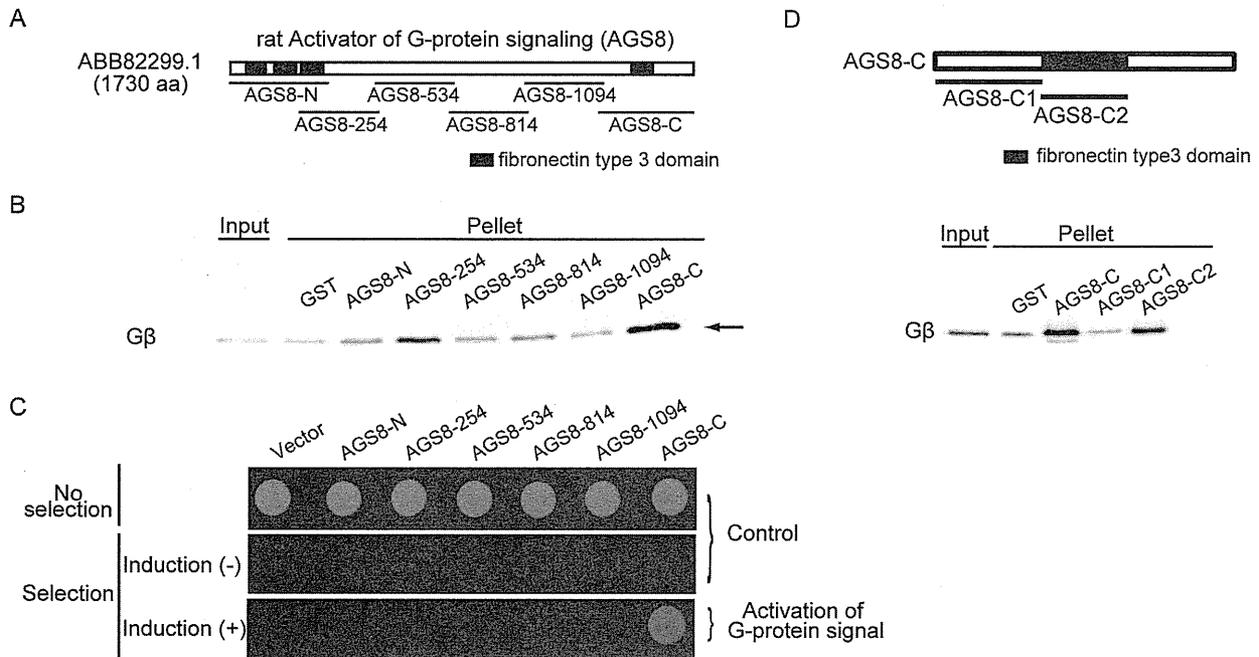
To further determine the region of interaction between AGS8 and Gβγ, we prepared multiple 29- to 30-amino-acid peptides, the sequences of which were based on the amino acids from A<sup>1494</sup> to W<sup>1730</sup> of the rat AGS8 (ABB82299) (Fig. 2A). In a screen of 11 peptides by the GST-pull-down assay, which covered the entire region from A<sup>1494</sup> to W<sup>1730</sup> of the rat AGS8, we found two peptides, CP1 (A<sup>1494</sup>PRNITVVAMEGCHSFVIVDWNKAIPGDV<sup>1522</sup>) and CP9 (S<sup>1508</sup>FVIVDWNKAIPGDVVVTGYLVYSASYEDFI<sup>1537</sup>) that effectively blocked the interaction of AGS8 with Gβγ in a dose-dependent manner (Fig. 2B). Quantitative analysis of immunoblots confirmed the dependence of the interaction of AGS8 with Gβγ on dose (Fig. 2C) and indicated that both peptides had similar IC<sub>50</sub>s (CP1: 6.73 × 10<sup>-6</sup> M; CP9: 2.77 × 10<sup>-6</sup> M). We first focused on CP9 and used this peptide as the AGS8-peptide in the following experiments.

### The Gβγ-interaction Site of AGS8 was Localized at the FN3 Domain of the C-terminus of AGS8

The present data suggested that the first 45 amino acids of the FN3 domain represented by CP1 and CP2 were critical for AGS8 to interact with Gβγ and mediate signal to downstream molecules. The importance of this region for AGS8-mediated signaling was further examined in the yeast cells in which growth was linked to G-protein activation [23]. A deletion mutant of AGS8-C, which lacked the first 45 amino acids of this domain, failed to activate G-protein signaling, indicating the importance of this region for mediating AGS8-Gβγ signaling in the cell (Fig. 3).

### The AGS8-peptide Inhibited Hypoxia-induced Internalization of Connexin 43 in Cardiomyocytes

In a previous study, we demonstrated that AGS8 was required for hypoxia-induced apoptosis of cardiomyocytes, which was associated with changes in the permeability of CX43 [13]. AGS8-Gβγ accelerates the internalization and degradation of channel



**Figure 1. Determination of  $G\beta\gamma$  interacting domain of the AGS8.** (A) Schematic diagram of rat AGS8 and the AGS8 domains synthesized as GST-fusion proteins. Each GST protein fused with the following segment of rat AGS8 (ABB82299) respectively. AGS8-N: M<sup>1</sup>-P<sup>370</sup>, AGS8-254: A<sup>254</sup>-R<sup>553</sup>, AGS8-534: S<sup>534</sup>-S<sup>833</sup>, AGS8-814: S<sup>814</sup>-R<sup>1113</sup>, AGS8-1094: H<sup>1094</sup>-D<sup>3280</sup>, AGS8-C: A<sup>1359</sup>-W<sup>1730</sup>. (B) GST-pulldown assay of AGS8 domains with  $G\beta_1\gamma_2$ . AGS8 domains synthesized as GST-fusion proteins (300 nM) were incubated with recombinant human  $G\beta_1\gamma_2$  (30 nM) in a total volume of 300  $\mu$ l at 4°C. Proteins were then adsorbed to a glutathione matrix and retained G-protein subunits identified by immunoblotting following gel electrophoresis. The representative of 5 independent experiments with similar results. (C) Bioactivity of AGS8 domains on G-protein activation in cell. The yeast strain expressing human  $G\alpha_s$  was transformed with AGS8 domains described in (A) into the pYES2-containing GAL1 promoter. The yeast strain was modified to grow without histidine on activation of G-protein. Induction(+): induction of translation of AGS8 domains by galactose. The representative of 4 independent experiments with similar results. (D) GST-pull down assay of AGS8-C segments with recombinant  $G\beta\gamma$ . (upper panel) Schematic diagram of AGS8-C and the segments synthesized as GST-fusion proteins. Each GST protein fused with the following segment of rat AGS8 (ABB82299) respectively. AGS8-C1: A<sup>1359</sup>-H<sup>1493</sup>, AGS8-C2: A<sup>1494</sup>-T<sup>1585</sup>. (lower panel) GST-pulldown assay of AGS8 segments with  $G\beta_1\gamma_2$ . AGS8 domains synthesized as GST-fusion proteins (300 nM) were incubated with recombinant human  $G\beta_1\gamma_2$  (30 nM) in a total volume of 300  $\mu$ l at 4°C. Proteins were then adsorbed to a glutathione matrix and retained G-protein subunits identified by immunoblotting following gel electrophoresis. The representative of 4 independent experiments with similar results. doi:10.1371/journal.pone.0091980.g001

protein CX43 under hypoxia, which results in decreased membrane permeability in the cardiomyocytes [13]. The change in localization and permeability of CX43 in the membrane is associated with hypoxia-induced apoptosis of the cardiomyocytes [14–16]. Therefore, we transferred the AGS8-peptide to cardiomyocytes and examined its effect on the internalization of CX43 induced by repetitive hypoxia (Fig. 4A).

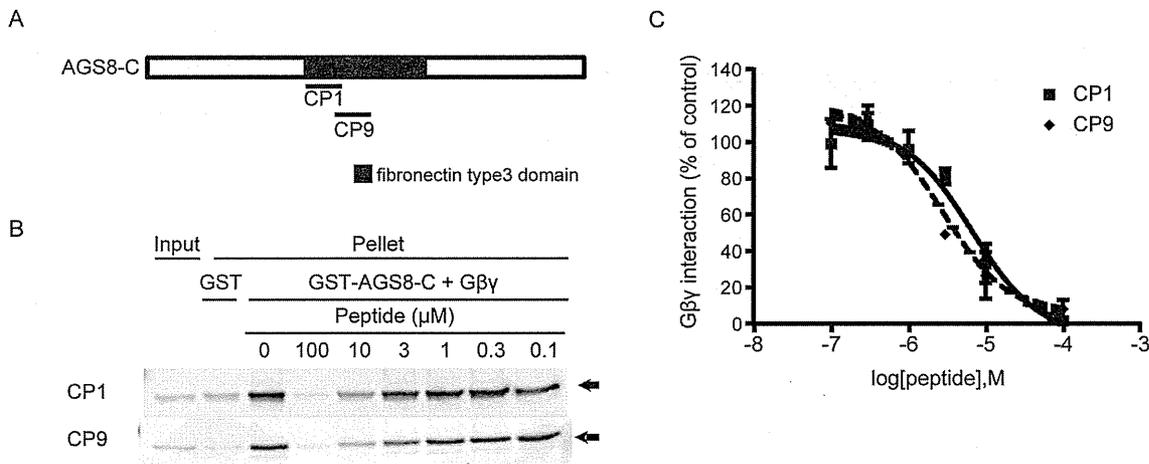
The peptide was delivered to the cardiomyocytes by chemical reagent as described in “experimental procedures”. Treatment of the cells with the chemical reagent and FITC-conjugated AGS8-peptide showed that the peptide was successfully delivered into the cardiomyocytes (Fig. 4B). The effect of the AGS8-peptide on internalization of CX43 was determined in the cardiomyocytes after hypoxic stress. CX43 was observed on the surface of the cardiomyocytes under normoxia (Fig. 4B), and its presence was decreased after exposure of the cardiomyocytes to repetitive hypoxia in the untransfected control cells and the cells exposed to transfection reagent alone (Fig. 4B, 4C). However, the AGS8-peptide dramatically blocked the internalization and degradation of CX43 induced by repetitive hypoxia (Fig. 4B, 4C). When the effect of FITC-conjugated AGS8-peptide was examined, FITC was observed in the cardiomyocytes under normoxia as well as hypoxia, and the hypoxia-induced internalization of CX43 was inhibited in FITC-positive cells (Fig. 4B).

### The AGS8-peptide Inhibited the Decrease in Permeability of Connexin 43 Under Hypoxia

Loss of CX43 from the cell surface decreases influx and efflux of small molecules passing through CX43, and this effect is associated with apoptosis of the cardiomyocytes under hypoxia [13–16]. We next examined the ability of AGS8-peptide to block the change in permeability of CX43 by analyzing by the flux of the fluorescent dye, Lucifer Yellow (LY), which passes through CX43 as previously demonstrated [13]. LY in the culture medium was incorporated into cardiomyocytes under normoxia. The flux of dye was decreased after exposure of the cells to repetitive hypoxia in the control group as well as in the group exposed to transfection reagent alone ( $31.1 \pm 4.4\%$ ,  $26.9 \pm 13.0\%$ , respectively) (Fig. 5). However, the AGS8-peptide blocked the hypoxia-induced decrease in permeability in a dose-dependent manner. This observation is consistent with the immunofluorescence studies, in which CX43 was observed to remain at the cell surface after repetitive hypoxia in the presence of AGS8-peptide (Fig. 4).

### The AGS8-peptide Protected Cardiomyocytes from Hypoxia-induced Apoptosis

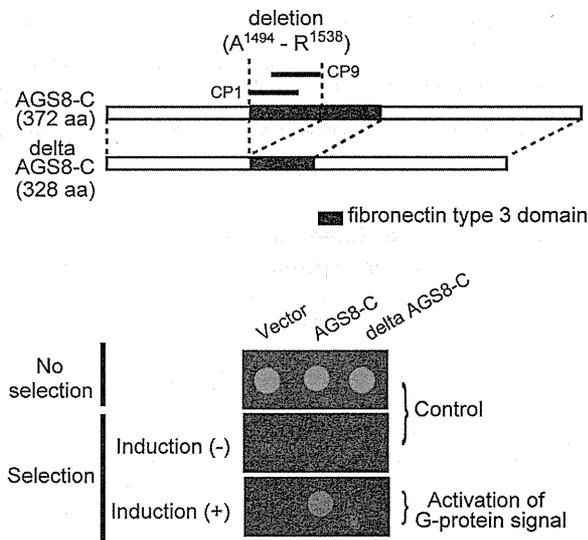
To examine the effect of AGS8 peptide on apoptosis of the cardiomyocytes, cultured cardiomyocytes were sequentially ex-



**Figure 2. Development of AGS8 peptide.** (A) Schematic diagram of AGS8-C and the AGS8-peptides (CP1 and CP9) which inhibited AGS8-Gβγ interaction. The AGS8-peptides were developed based on amino acid sequences of the Gβγ interaction domain of AGS8. CP1 and CP2 represented A<sup>1494</sup>-V<sup>1522</sup> and S<sup>1508</sup>-I<sup>1537</sup> of rat AGS8 (ABB82299) respectively. (B) The example of GST-pulldown assay of GST-AGS8-C with Gβ<sub>1</sub>γ<sub>2</sub>. GST-fusion protein (100 nM) was incubated with recombinant human Gβ<sub>1</sub>γ<sub>2</sub> (10 nM) in the presence of AGS8-peptides (CP1 or CP9). Proteins were then adsorbed to a glutathione matrix and retained G-protein subunits identified by immunoblotting following gel electrophoresis. The representative of 6 independent experiments with similar results. (C) The densitometric analysis of GST-pulldown assay of GST-AGS8-C with Gβ<sub>1</sub>γ<sub>2</sub> in the presence of AGS8-peptides. n=6 with peptides of ~97% HPLC purity. doi:10.1371/journal.pone.0091980.g002

posed to 1% oxygen for 6 h, then to 12 h of normoxia to induce hypoxia-mediated cell death (Fig. 6A) [13]. Hypoxia/reoxygenation markedly increased the number of apoptotic cardiomyocytes,

as determined by TUNEL or immunostaining of cleaved caspase-3, in the untransfected control cells and those exposed to transfection reagent alone (Fig. 6B and 6C). However, AGS8-peptide successfully inhibited hypoxia-induced apoptosis, indicating a protective effect in cardiomyocytes. Additionally, the data indicated that, under normoxia, the AGS8-peptide did not influence apoptosis or the permeability of CX43 (Fig. 6B and 6C).

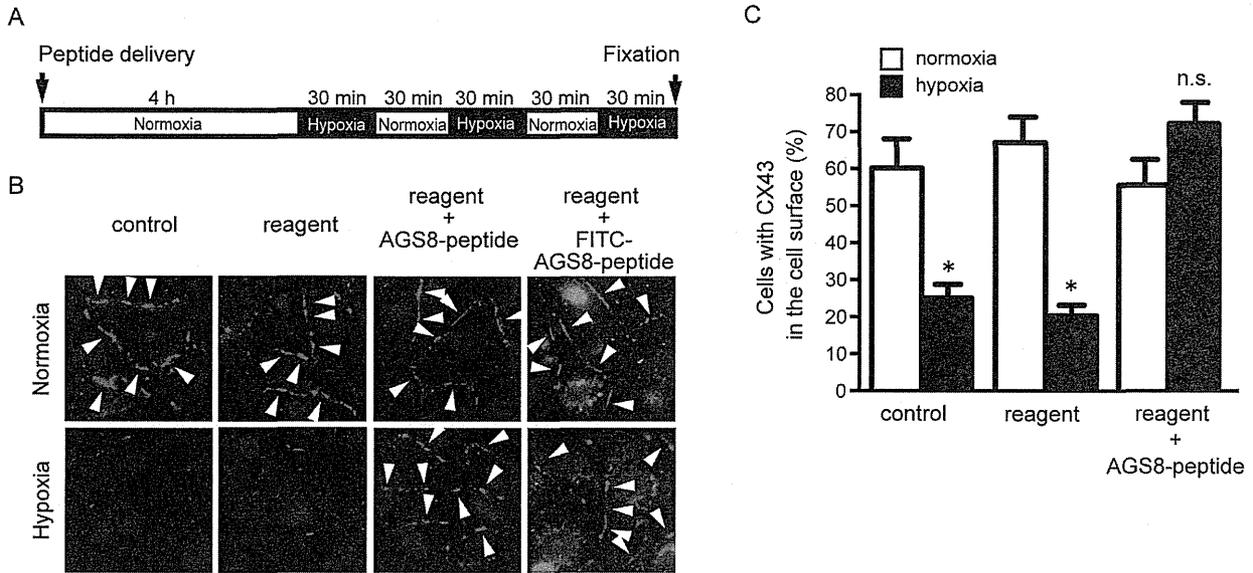


**Figure 3. Effect of deletion of 45 amino acids of fibronectin type 3 domain.** (A) Schematic diagram of C-terminal of AGS8 (AGS8-C) and the deleted mutant of AGS8-C (delta AGS8-C) lacking the first 45 amino acids of fibronectin type 3 domain, that are A<sup>1494</sup> to R<sup>1538</sup> of rat AGS8 (ABB82299). (B) Bioactivity of AGS8C and delta AGS8C on G-protein activation. The yeast strain expressing human Gαs was transformed with AGS8-C and delta AGS8-C in the pYES2-containing GAL1 promoter. The yeast strain was modified to grow without histidine on activation of G-protein. Induction (+): induction of translation of AGS8 domains by galactose. The representative of 4 independent experiments with similar results. doi:10.1371/journal.pone.0091980.g003

### Advantage of the AGS8-peptide for Targeted Disruption of the Gβγ Pathway

The data presented thus far indicated that the AGS8-peptide blocked the AGS8-Gβγ interaction and the signaling events the downstream of AGS8-Gβγ. We next asked whether inhibition of the Gβγ-mediated signal generally had a cardioprotective effect as was observed with the AGS8-peptide. Gallein is an inhibitor of Gβγ-mediated signaling that occupies a “common” interaction surface of Gβγ and inhibits the interaction of Gβγ-regulated proteins with Gβγ [25]. Thus, gallein is expected to block a wide range of Gβγ signals in cells, including CX43 regulation mediated by AGS-Gβγ. We previously demonstrated that gallein completely inhibits hypoxia-induced internalization of CX43, but not at 1 μM [13].

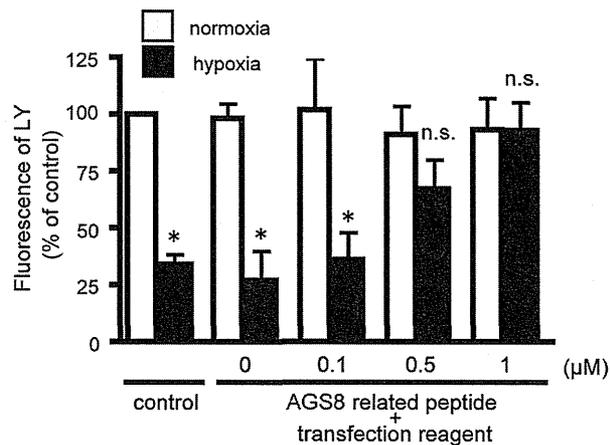
Here, we first examined the influence of the AGS8-peptide and gallein on the viability of cells. Cardiomyocytes were incubated with gallein for 24 h, and their viability was determined by MTT assay. Even the lowest concentration of gallein (1 μM) caused damage in the cardiomyocytes, indicating that broad inhibition of Gβγ did not have a protective effect (Fig. 7). In contrast, the AGS8-peptide did not show cytotoxicity at 1 μM, the concentration that completely blocked the AGS8-Gβγ-mediated signal event. This suggests that the AGS8-peptide is a promising candidate for protection of cardiomyocytes from hypoxia-mediated apoptosis.



**Figure 4. Effect of AGS8-peptide on localization of connexin 43 (CX43) of cultured cardiomyocytes.** (A) Cardiomyocytes were exposed 3 times to 30 min of hypoxia (1% oxygen) intermittent with 30 min of reoxygenation 4 h after (without or with) transfection of AGS8-peptide (1  $\mu$ M) or FITC-conjugated AGS8-peptide (1  $\mu$ M). (B) Localization of CX43 in the cardiomyocytes under normoxia and hypoxia. The figures demonstrated in the triple color of CX43 (red, arrow), nuclei (DAPI, blue) and FITC-conjugated AGS8-peptide (green). The representative of 5 independent experiments with similar results. (C) The number of cardiomyocytes expressing CX43 in the cell surface were counted. Please note that  $\sim$  90% cells were detectable at the point of fixation. Data are represent 170–260 cells from 5 of independent experiments. \*,  $p < 0.05$  vs normoxia group. doi:10.1371/journal.pone.0091980.g004

## Discussion

AGS8-peptide, the sequence of which was derived from the amino-acid sequences of the G $\beta$  $\gamma$ -binding domain of AGS8,

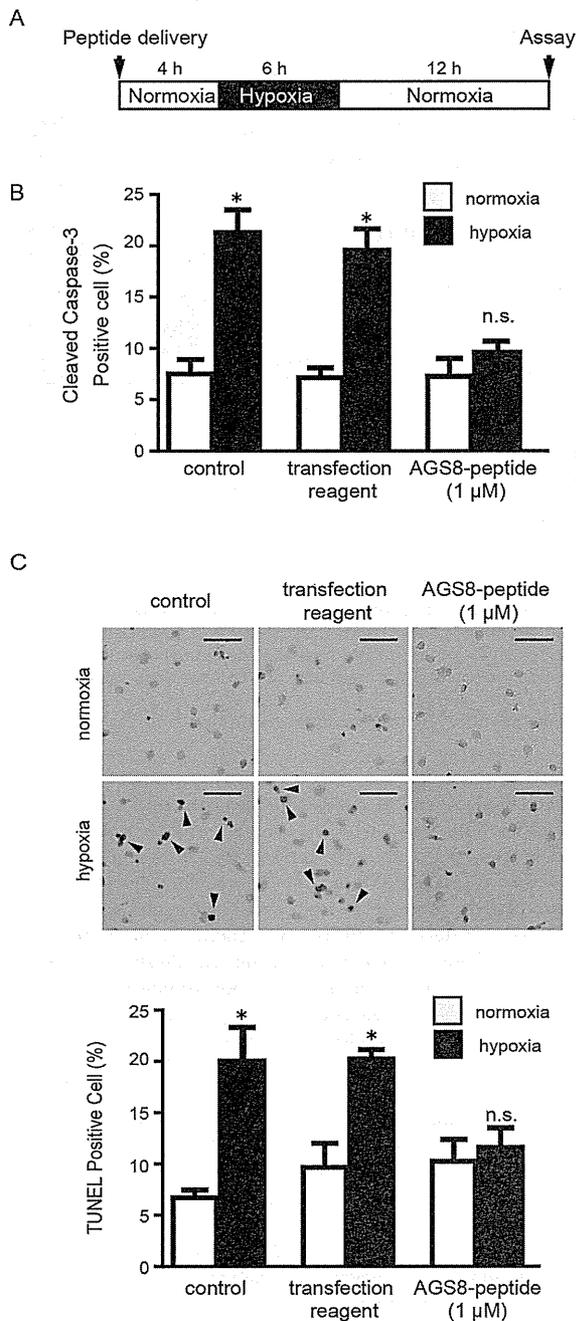


**Figure 5. Uptake of connexin selective fluorescence dye, Lucifer Yellow (LY) to the cardiomyocytes.** Cells were incubated with 1 mM of LY for 45 min in normal culture medium. Excess LY was removed and cells were rinsed with PBS. The fluorescence of LY was evaluated by inverted fluorescence microscope. The fluorescence of LY was quantified by the intensity of fluorescence of 10 randomly selected fields. The non-selective binding and/or incorporation of LY was determined fluorescence in the presence of a connexin hemichannel blocker, 50  $\mu$ M of Lanthanum which was added 30 min prior to LY. \*,  $p < 0.05$  vs cells in normoxia; n.s., not statistically significant. N = 4 from 4 independent experiments. doi:10.1371/journal.pone.0091980.g005

blocked the association of AGS8 with G $\beta$  $\gamma$  and inhibited AGS8-mediated events under hypoxia. Notably, AGS8-peptide inhibited the change in permeability of cell-surface CX43 and the apoptosis of cardiomyocytes. AGS8-peptide did not show cytotoxicity under normoxia, in contrast to the small molecule gallein, which produced deleterious effects by general inhibition of G $\beta$  $\gamma$ -signaling. These data indicate that the AGS8-G $\beta$  $\gamma$  complex plays a pivotal role in triggering the hypoxia-induced apoptosis of cardiomyocytes. Furthermore, they suggest an advantage of targeted disruption of G-protein signaling by the AGS-based peptide in protecting cardiomyocytes from hypoxia-induced apoptosis.

Several peptides have been developed to manipulate the broad signal initiated by accessory proteins for G-proteins. For example, an inhibitor of accessory proteins for heterotrimeric G-protein has been developed for RGS proteins. RGS proteins share 120–130 amino acids of an RGS-homology domain, which interact with the G $\alpha$  subunit and accelerate GTPase activity [26,27]. RGS-peptides, which were designed on the basis of the X-ray structure of the G $\alpha$ i switch I region of RGS4-G $\alpha$ i, successfully modulate muscarinic receptor-regulated potassium currents in atrial myocytes [28]. RGS-peptides were initially designed to mimic the surface of G-protein to inhibit GAP activity, which produced the potential to suppress many events mediated by a variety of RGS proteins. As another example, a peptide, the sequence of which is based on G-protein regulatory (GPR) or GoLOCO motifs, is a cassette of 20–25 amino acids that stabilizes the GDP-bound conformation of G $\alpha$  and clearly inhibits G $\alpha$ i activation in vitro, suggesting its potential as an inhibitor of general G $\alpha$ i signaling in cells [29].

Alternatively, the AGS8 peptide was designed on the basis of the AGS-G $\beta$  $\gamma$  interface, with the objective of suppressing the specific signal evoked by AGS-G $\beta$  $\gamma$  for all G $\beta$  $\gamma$  signaling. It has been demonstrated that G $\beta$  $\gamma$ -interacting proteins share an overlapping

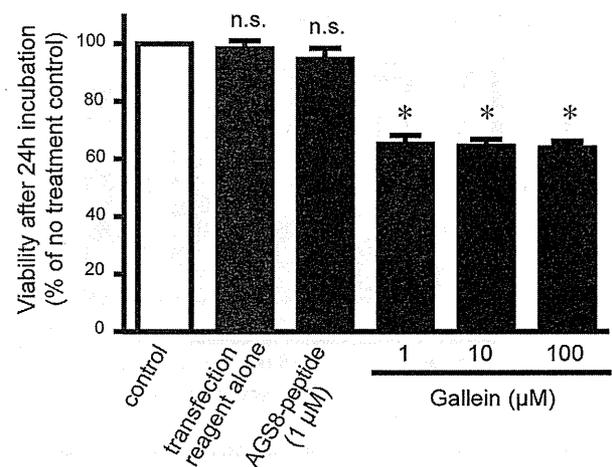


**Figure 6. Effect of AGS8 peptide on hypoxia-induced apoptosis of cardiomyocytes.** (A) Neonatal cardiomyocytes were exposed to hypoxia (1% oxygen) or normoxia as indicated duration without or with introduction of AGS8-peptide. Apoptosis was assessed by immunofluorescent detection of the active form of caspase-3 (cleaved caspase-3) (B) or TUNEL stain (C) as described in the experimental procedures. (C) Upper panel indicates representative apoptotic (dark blue, arrow) and non-apoptotic cells (red) after TUNEL staining. Scale bars indicate 100 μm. Approximately 3000 cells of 10 independent fields were counted for each experiments. A separate experiment indicated that AGS8-peptide did not influence the level of AGS8 within the 4-h treatment (1.0 μM peptide; 98.2±7.3% versus no reagent alone group, not statistically significant, *n* = 4). \*, *p* < 0.05 vs cells in normoxia; *n.s.*, not statistically significant. *N* = 5 from 5 independent experiments. doi:10.1371/journal.pone.0091980.g006

interface on the surface of Gβγ [30,31]. We previously demonstrated that the Gβγ-interacting surface of AGS8 includes the shared-site [32]. However, the entire Gβγ-interacting surface of AGS8 may include an additional interface, which is required to form an AGS8-specific signal complex. Our results indicated that 45 amino acids were required to evoke cell signaling by the AGS-Gβγ complex. The amino acid sequence of this domain did not have similarity to other known Gβγ interfaces [33–35], suggesting this sequence represents the AGS8-specific interface that forms this particular complex. In fact, the AGS8-peptide designed to recognize this region successfully inhibited formation of the AGS8-Gβγ complex and subsequent cell events mediated by AGS8-Gβγ. However, currently, there is not enough information to determine whether the AGS8-peptide covers the common Gβγ interface or influences the interaction of Gβγ with other molecules. These issues are to be investigated elsewhere.

AGS-Gβγ-mediated signaling is required for hypoxia-induced apoptosis of cardiomyocytes [13]. Gβγ is known to conduct pro-apoptotic signaling via p38MAPK, JNK, or PLCβ, whereas anti-apoptotic signaling is also mediated by Gβγ via PI3K-Akt or ERK depending on type of cell and stimulus [36–39]. Although the involvement of these Gβγ-mediated pathways in the AGS-Gβγ pathway or other players in the AGS-Gβγ protein complex are yet to be determined, the current data indicate the presence of a critical signaling pathway mediated by the AGS8-Gβγ complex leading to apoptosis in cardiomyocytes.

The channel protein CX43 plays a role in the apoptotic process by regulating the permeability of small molecules, including adenosine 5'-triphosphate, adenosine diphosphate, adenosine, cAMP, inositol-1,4,5-triphosphate, glutamate, and glutathione [14,15,40–42]. In response to hypoxia, AGS8 organizes the complex that includes Gβγ, CX43, and possibly kinases that initiate phosphorylation of CX43 [13]. Multiple phosphorylation sites on CX43 are regulated by specific kinases under ischemic



**Figure 7. Effect of AGS8-peptide or Gallein on the viability of cardiomyocytes determined by MTT assay.** Neonatal cardiomyocytes were cultured for 24 h with AGS8-peptide or Gallein. Gallein occupied a “common” interaction surface of Gβγ and inhibited its interaction with Gβγ-regulated proteins. \*, *p* < 0.05 vs cells in normoxia; *n.s.*, not statistically significant. *N* = 5 from 5 independent experiments. doi:10.1371/journal.pone.0091980.g007

conditions, and each phosphorylation critically influences the permeability and localization of CX43 in the sarcolemma [43,44]. The inhibition of internalization of CX43 by AGS8-peptide may suggest that AGS8-peptide blocked the recruitment of components into the complex and/or phosphorylation of CX43 within the complex. AGS8 may play a role in separating the hypoxic/ischemic cardiomyocytes from healthy tissue by disconnecting the gap junction, which can contribute to arrhythmia and contraction failure during ischemia. Although the validity of this hypothesis is yet to be tested in confirmed or animal models, our observations support this possibility.

Myocardial ischemia activates multiple cascades that initiate intracellular ionic and chemical changes leading cell to death [17,18]. Although great efforts have been made over many years, therapeutic approaches to ischemic heart disease still need further development [45,46]. We previously demonstrated that AGS8-G $\beta\gamma$  signaling was activated under hypoxia and did not constitutively stimulate the apoptotic pathway, because the pro-apoptotic effects of AGS were not observed in cells cultured under normoxia [13]. In fact, AGS8-peptide effectively protected cardiomyocytes from hypoxia-mediated cell death, but did not cause cell damage under normoxia. Thus, AGS8-peptide has the potential to save a subpopulation of cardiomyocytes exposed to hypoxia without producing damage to the entire myocardium. This is an advantage of targeted disruption of specific signaling by AGS8-peptide, and it stands in contrast to the deleterious effects caused by general inhibition of G $\beta\gamma$  signaling with gallein.

Peptide-based reagents designed from the sequences of accessory proteins for heterotrimeric G-protein have potential to manipulate alternative signaling events distinct from GPCR-mediated G-protein signaling. At this stage, the potential of peptide-based reagents, including AGS8-peptide, remains limited by their ability to penetrate the membrane, their stability, and the modes of delivery available for their use as clinical drugs. The strategies for effective delivery of therapeutic peptides into the cell

include conjugation with cell-penetrating peptides, incorporation into polymeric nanoparticles, and virus-mediated release of peptides [47–49]. Each strategy still has challenges with respect to the efficiency or selectivity of delivery as well as safety in healthy tissues or cells [47,49,50]. Although intracellular use of peptide targeting is not yet a common practice, delivery systems are continuously being developed, which will increase the potential for use of peptide-targeting therapy. We have demonstrated the possibility of regulating intracellular signaling mediated by accessory proteins for heterotrimeric G-protein, which could be a starting point for development of peptides or compounds to regulate AGS8-G $\beta\gamma$  signaling.

Targeted disruption of the interaction of G-protein with accessory proteins for heterotrimeric G-protein is a promising therapeutic strategy because these molecules mediate critical signaling distinct from the receptor-mediated pathway. In this study, we demonstrated that AGS8-peptide could be part of a novel therapeutic approach for the protection of ischemia in the heart that has enhanced specificity and fewer side effects. Although efforts to intervene in G-protein signaling have been focused on the interface of the GPCR-heterotrimeric G-protein, our data highlight the advantages of accessory proteins for heterotrimeric G-protein as alternative therapeutic targets in human diseases.

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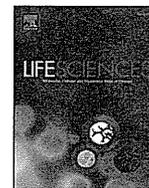
## Author Contributions

Conceived and designed the experiments: M. Sato. Performed the experiments: M. Sato MH HS YY. Analyzed the data: M. Sakima AAM TF UY SO. Contributed reagents/materials/analysis tools: UY SO TF YI. Wrote the paper: M. Sato.

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## Geranylgeranylacetone protects the heart via caveolae and caveolin-3



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### ABSTRACT

**Aims:** Geranylgeranylacetone (GGA) is commonly utilized to protect the gastric mucosa in peptic ulcer disease. Recently GGA has been shown to protect the myocardium from ischemia/reperfusion by activating heat shock proteins. However, the exact mechanism as to how GGA activates these protective proteins is unknown. Caveolae and caveolin-3 (Cav-3) have been implicated in ischemia, anesthetic, and opioid induced cardiac protection. Given the lipophilic nature of GGA it is our hypothesis that GGA induced cardiac protection requires caveolae and Cav-3.

**Main methods:** We used an in vivo mouse model of ischemia–reperfusion injury and performed biochemical assays in excised hearts.

**Key findings:** GGA treated control mice revealed increased caveolae formation and caveolin-3 in buoyant fractions, mediating heat shock protein 70 activation. Furthermore, control mice treated with GGA were protected against ischemia/reperfusion injury whereas Cav-3 knockout (Cav-3 KO) mice were not. Troponin levels confirmed myocardial damage. Finally, Cav-3 KO mice treated with GGA were not protected against mitochondrial swelling whereas control mice had significant protection.

**Significance:** This study showed that caveolae and caveolin-3 are essential in facilitating GGA induced cardiac protection by optimizing spatial and temporal signaling to the mitochondria.

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### Introduction

Geranylgeranylacetone (GGA), an acyclic polyisoprenoid, is commonly used as an oral anti-ulcer medication in Asia. This compound has been shown to be effective in protecting the gastric mucosa against insults without affecting gastric acid secretion (Fujimoto et al., 1982; Murakami et al., 1981). Oral GGA has been demonstrated to have protective effects on myocardial ischemia/reperfusion injury within the rat heart (Ooie et al., 2001). Yamanaka et al. suggested that GGA-induced cardiac preconditioning exerted protective effects on myocardial ischemia/reperfusion injury by mediating the activation of protein kinase C and heat shock protein (HSP) (Yamanaka et al., 2003). Further investigations demonstrated that GGA also prevented cellular endothelial damage (Zhu et al., 2005), diminished apoptosis and preserved mitochondrial respiratory function (Shinohara et al., 2007).

Structural scaffolding domains commonly organize signal transduction molecules and receptors. Caveolae are small membrane invaginations of the plasma membrane that are enriched in sphingolipids, cholesterol, and lipid rafts (Lisanti et al., 1994; Palade, 1953; Patel et al., 2008). Caveolins are the structural proteins of caveolae and are present in three isoforms, caveolin (Cav)-1, -2, and -3 (Lisanti et al., 1994; Patel et al., 2008). Additionally, many signaling molecules are known to localize in caveolae and interact with the scaffolding domain of caveolin. We have recently shown that both caveolae and Cav-3 were essential for ischemic (Horikawa et al., 2008; Tsutsumi et al., 2008), anesthetic (Horikawa et al., 2008), and opioid (Tsutsumi et al., 2010b) induced cardiac protection. It is our hypothesis that caveolin-3 and caveolae are also essential in GGA induced cardiac protection.

### Materials and methods

#### Animals

All animals were treated in compliance with the Guidelines for Proper Conduct of Animal Experiment and Related Activities (Ministry of Education, Culture, Sports, Science and Technology of Japan) and the Guideline for Care and Use of Lab Animals at the University of

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Tokushima. Animal use protocols were approved by the Animal Care and Use Committee, the University of Tokushima. Male C57BL/6 mice (8–10 weeks old, 21–26 g body weight) were purchased from Japan SLC and Cav-3 knockout (Cav-3 KO) mice were created as reported previously (Hagiwara et al., 2000). Animals were randomly assigned into treatment groups by an independent observer. The animals were kept on a 12 hour light–dark cycle in a temperature-controlled room. GGA was provided by Eisai Co., Tokyo, Japan and was given by gavage at a dose of 200 mg/kg (dissolved with 0.4% lecithin in deionized water). Mice in the control group were given the same dose of vehicle.

#### Electron microscopy

Wild-type mice were given GGA or vehicle by oral gavage. Twenty four hours later, whole hearts were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 2 h at room temperature, post-fixed in 1% OsO<sub>4</sub> in 0.1 M cacodylate buffer (1 h), and uranyl acetate, dehydrated in a graded series of ethanol solutions, and embedded in epon epoxy resin. Sections were cut with a Reichert Ultracut E Ultramicrotome (Leica Microsystems, Wetzlar, Germany) and observed with an electron microscope (Hitachi H7650, Hitachi Co., Tokyo, Japan). Random sections were taken by an electron microscopy technician blinded to the treatments.

#### Sucrose density membrane fractionation

We performed whole left ventricle sucrose density membrane fractions as reported previously (Tsutsumi et al., 2008). Fraction samples 4–12 were used in immunoblot analyses. We defined fractions 4–6 as lipid rich–buoyant membrane fractions enriched in caveolae and proteins associated with caveolae. Fractions 9–12 were defined as non-buoyant fractions.

#### Immunoblot analysis

Proteins in whole left ventricle or membrane fractions were separated by SDS-PAGE 10% polyacrylamide precast gels (Bio-Rad Laboratories) and transferred to a polyvinylidene difluoride membrane by electroelution. Membranes were blocked in PBS containing 2.0% nonfat dry milk and incubated with primary antibody overnight at 4 °C. Bound primary antibodies were visualized using secondary antibodies conjugated with horseradish peroxidase from Santa Cruz Biotechnology (Santa Cruz) and ECL reagent from Amersham Pharmacia. All displayed bands migrated at the appropriate size, as determined by comparison to molecular weight standards (Santa Cruz Biotechnology).

#### Immunoprecipitation

Immunoprecipitation was performed using Protein A Sepharose CL-4B (GE Healthcare) as described previously (Hirose et al., 2011). Buoyant fraction samples were incubated with primary antibody for 3 h at 4 °C, immunoprecipitated overnight with protein-agarose at 4 °C, and then centrifuged for 5 min at 13,000 g. Protein-agarose pellets were washed 3 times. Wash buffer was removed and sample buffer was added, and then boiled for 5 min at 95 °C for immunoblotting.

#### Ischemia–reperfusion protocol and experimental groups

Cav-3 KO and control mice were randomly assigned to receive GGA or vehicle 24 h before ischemic injury. Mice were anesthetized with pentobarbital sodium (80 mg/kg i.p.) and mechanically ventilated by using a pressure-controlled ventilator (TOPO Ventilator, Kent Scientific) as described before (Tsutsumi et al., 2006, 2007). Core temperature was maintained with a heating pad and ECG leads were placed to record heart rate. The hemodynamic effects were measured through the right carotid artery cannulation with a 1.4F Mikro-tip pressure transducer

(Model SPR-671, Millar Instruments), which was connected to an amplifier (Model TC-510, Millar Instruments) for determination of heart rate, arterial blood pressure, and rate pressure product.

Lethal ischemia was produced by occluding the left coronary artery with a 7–0 silk suture placed with a tapered BV-1 needle (Ethicon) for 30 min. After 30 min of occlusion, the ligature was released and the heart was reperfused for 2 h. After reperfusion, mice were heparinized, and the coronary artery was again occluded. The area at risk (AAR) was determined by staining with 1% Evans blue (1.0 mL, Sigma). The heart was immediately excised and cut into 1.0-mm slices (McIlwain tissue chopper; Brinkmann Instruments). Each slice of left ventricle (LV) was then counterstained with 2,3,5-triphenyltetrazolium chloride (Sigma). After overnight storage in 10% formaldehyde, slices were weighed and visualized under a microscope (SZ61-TR, Olympus) equipped with a charge coupled device camera (DXM 1200F, Nikon). The images were analyzed (Image-Pro Plus, Media Cybernetics), and area at risk (AAR) and infarct size (IS) were determined by planimetry as previously described (Tsutsumi et al., 2008; Horikawa et al., 2008). Cardiac troponin I (cTnI) levels in the serum were measured using a High Sensitivity Mouse Cardiac Troponin-I ELISA Kit (Life Diagnostics) as described before (Tsutsumi et al., 2010a).

#### Mitochondrial isolation and swelling assay

Mice were anesthetized and hearts were harvested, immediately rinsed with PBS, and placed in homogenizer containing 4 mL sucrose buffer A (300 mM sucrose, 10 mM Tris–HCl, 2 mM EGTA and 5 mg/mL BSA, pH 7.4) on ice. Hearts were homogenized using 10 strokes and homogenate centrifuged at 2000 ×g for 2 min at 4 °C to remove cell debris. The supernatant was further centrifuged at 10,000 ×g for 30 min at 4 °C to sediment impure mitochondria. Mitochondrial pellet was purified and washed as described previously (Fridolfsson et al., 2012). 200 μL of mitochondria in sucrose buffer B (300 mM sucrose, 10 mM Tris–HCl, pH 7.4) was loaded on to 96-well plate and challenged with 100 μM CaCl<sub>2</sub> (2 mg/mL protein concentration). Absorbance was measured every 2 s for 600 measurements at 520 nm using a VarioScan Flash spectrophotometer (Thermo Scientific). 250 nM cyclosporine A (Sigma) pretreatment of mitochondria was used to inhibit CaCl<sub>2</sub> induced mitochondrial swelling to confirm the mitochondrial permeability transition pore (mPTP) dependence of calcium-induced swelling.

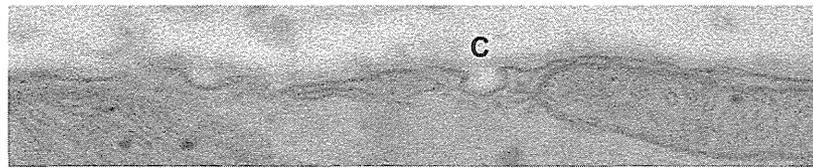
#### Statistical analysis

Determination of all data was performed blinded to experimental groups for the observers. Data were analyzed using Prism 6.0 (GraphPad Software, Inc.). Statistical analyses for Western blot studies were performed using Student's *t*-tests. When one-way ANOVA was needed, the Bonferroni post-hoc test was used. All data are expressed as mean ± SD. Statistical significance was defined as *P* < 0.05

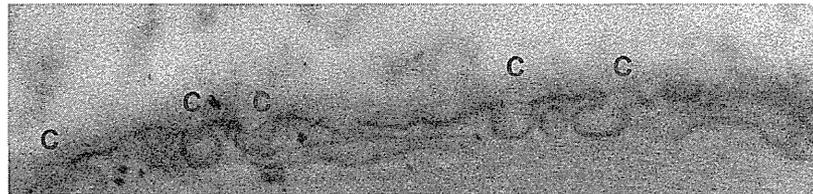
#### Results

The effect of GGA on cardiac membrane caveolae was assessed by pre-treating mice with and without GGA and performing electron microscopy analysis. Representative electron microscopy images show that pretreatment of GGA increased membrane caveolae compared to vehicle treated control animals (Fig. 1). However, Cav-3 KO mice had significantly decreased caveolae with no significant change after GGA treatment (data not shown). To verify these findings, hearts from GGA and vehicle control animals were fractionated on a discontinuous sucrose gradient and analyzed for protein content. GGA increased both Cav-3 protein expression and HSP 70 expression in buoyant fractions 4–6, which are associated with caveolae (Fig. 2A and 2B). Additionally, immunoprecipitation experiments showed that GGA treated mice resulted in HSP 70 associated with Cav-3 in buoyant fractions (Fig. 2C), suggesting that Cav-3 is essential for HSP 70 localization and activation.

## Control



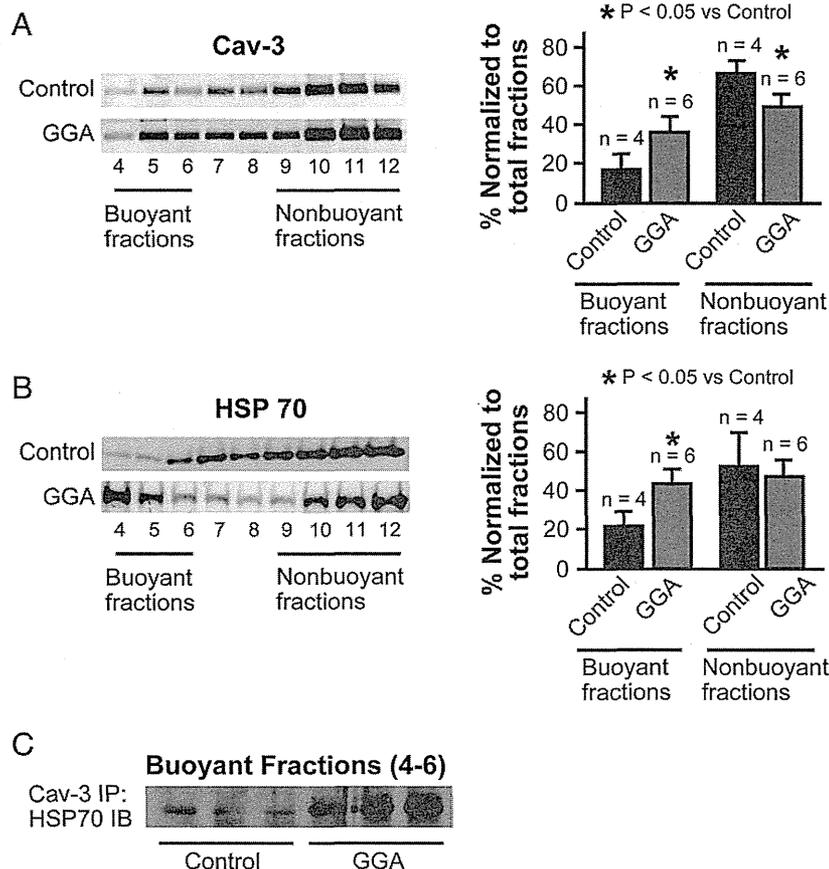
## GGA



**Fig. 1.** Geranylgeranylacetone (GGA) increases myocardial caveolae. Electron microscopy of control and GGA treated mouse myocardium revealed a significant increase in caveolae (red C) at the sarcolemmal surface in GGA treated mice.

We found no significant differences between wild-type and Cav-3 KO mice in heart rate, blood pressure, or rate pressure product with and without GGA administration at the baseline period (Table 1). The area at risk was calculated as a percentage of the left ventricle mass

and was similar between groups (Fig. 3A). Twenty-four hours after GGA administration, a significant reduction in myocardial ischemia/reperfusion injury was observed compared with vehicle control in the wild-type mice ( $29.0 \pm 3.7\%$  [ $n = 8$ ] vs.  $44.1 \pm 9.3\%$  [ $n = 10$ ],



**Fig. 2.** Lysed and fractionated hearts on a sucrose density gradient. Fractions 4–6 were considered buoyant and 9–12 non-buoyant fractions. \* $P < 0.05$  treated group versus control. A) and B) Fractions were collected and probed for both caveolin-3 (Cav-3) and heat shock protein 70 (HSP 70). Significant buoyant fraction localization of Cav-3 and HSP 70 was observed in geranylgeranylacetone (GGA) treated groups. C) The resulting lysates were immunoprecipitated (IP) with Cav-3 antibody and immunoblotted (IB) for HSP 70;  $n = 3$  animals per group. There was an increased association of Cav-3 with HSP 70 after GGA administration.

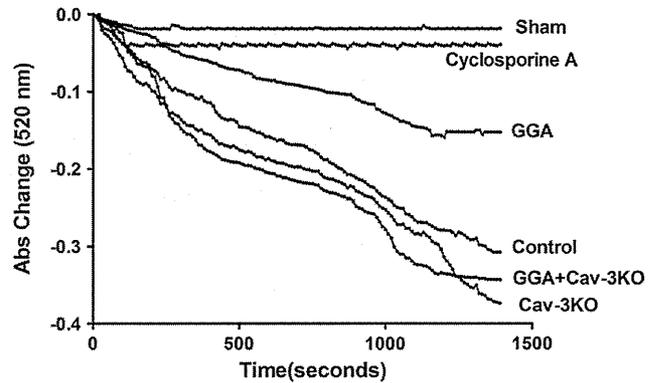
**Table 1**  
Hemodynamics parameters at baseline period.

	Control	GGA	Cav-3 KO	GGA + Cav-3 KO
Heart rate (/min)	421 ± 33	437 ± 21	413 ± 35	426 ± 37
MAP (mm Hg)	73 ± 6	69 ± 8	71 ± 4	70 ± 3
RPP (beats·min <sup>-1</sup> ·mm Hg·10 <sup>3</sup> )	30.5 ± 3.7	30.0 ± 3.3	29.4 ± 4.0	29.8 ± 2.3

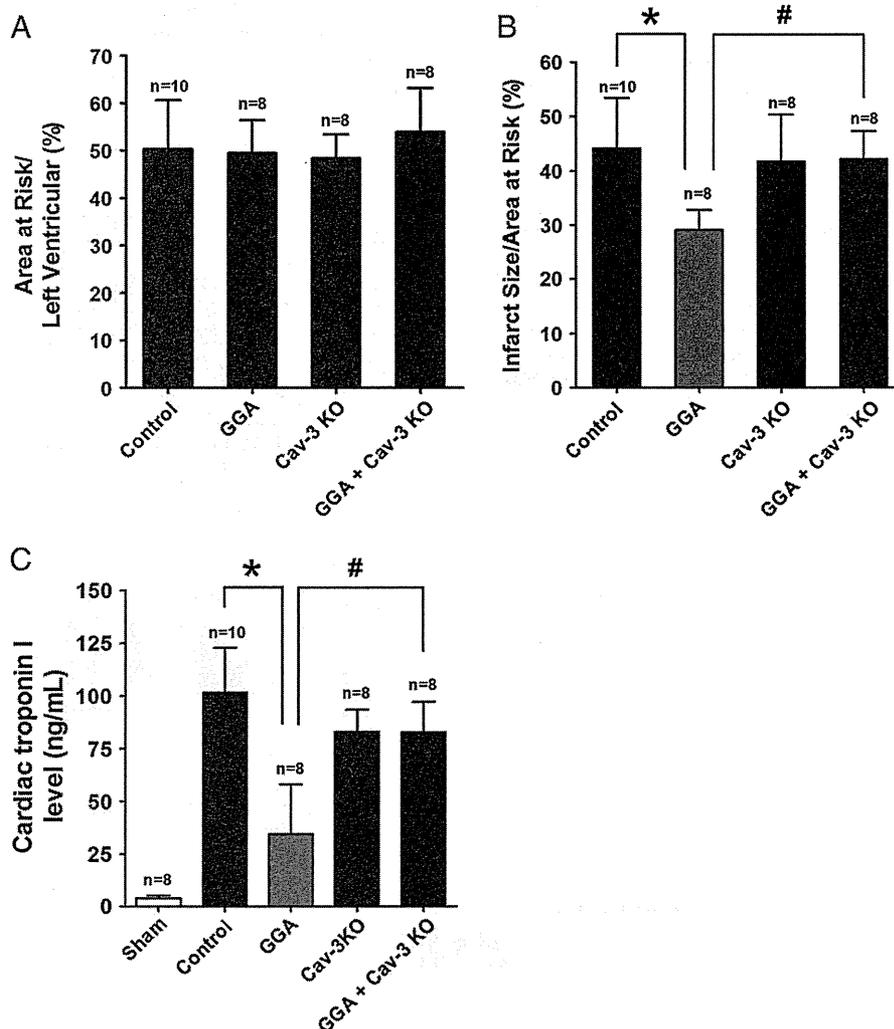
MAP, mean arterial pressure; RPP, rate-pressure product. Data are expressed as mean ± SD.

respectively). However, in Cav-3 KO mice, this cardiac-protective effect of GGA was abolished ( $42.1 \pm 5.2\%$  [ $n = 8$ ], Fig. 3B). Additionally, we confirmed these effects by measuring serum troponin I level, a marker of cardiac myocyte damage (Fig. 3C).

GGA reduces  $\text{Ca}^{2+}$ -induced swelling in isolated mouse heart mitochondria (Fig. 4). In wild-type mice, the addition of  $100 \mu\text{M}$   $\text{Ca}^{2+}$  caused a significant decrease in absorbance, indicating mitochondrial swelling.  $\text{Ca}^{2+}$  induced swelling was inhibited by cyclosporine A, a mPTP inhibitor, as reported previously (Tsutsumi et al., 2011). Under these conditions, GGA significantly attenuated  $\text{Ca}^{2+}$  induced swelling compared with the control; however, these effects were abolished in Cav-3 KO mice.



**Fig. 4.** Geranylgeranylacetone (GGA) inhibits mitochondrial swelling caused by ischemia-reperfusion injury. GGA treated mitochondria were isolated and revealed substantially less mitochondrial swelling compared to non-treated and Cav-3 KO mice with and without GGA when exposed to calcium chloride. Cyclosporine A was used as a control to inhibit  $\text{CaCl}_2$  induced mitochondrial swelling to confirm the mitochondrial permeability transition pore (mPTP) dependence of calcium-induced swelling ( $n = 3$ ).



**Fig. 3.** Geranylgeranylacetone (GGA) protects the mouse myocardium from ischemic injury. A) Area at risk was calculated as a percentage of the left ventricle and revealed no significant differences between all groups. B) GGA was able to induce significant protection in control animals, although no protection was observed in Cav-3 KO mice. C) Cardiac troponin I, a marker of myocardial damage also revealed a significant decrease in GGA treated control mice, but no effect in Cav-3 KO mice. \*  $P < 0.05$ ; #  $P < 0.05$ .

## Discussion

GGA, an anti-ulcer medication has been shown to have cardio-protective effects and modulate heat shock proteins, however the exact mechanisms on how this oral medication is able to enter the myocardial cell is unknown. We show that caveolae and Cav-3 are essential for GGA uptake and cardiac protection, by mediating the activation of HSP 70. Furthermore, these effects are not only mediated by caveolae and caveolins, but also directly affect mitochondrial function. These data suggest that GGA utilizes caveolae and caveolins in order to navigate the complex cell structure and facilitate protective signaling via mitochondria.

Caveolae are cholesterol and sphingolipid enriched invaginations of the plasma membrane that play a role in physiological functions and vital to cardio-protective mechanisms. In some cases, caveolae and caveolins regulate receptor stability, myocardial hypertrophy (Horikawa et al., 2011), signaling (Lisanti et al., 1994; Ostrom et al., 2001, 2002; Patel et al., 2007; Steinberg and Brunton, 2001), calcium homeostasis (Fujimoto, 1993), and endocytosis (Anderson, 1993). Recently, a decreased number of myocardial caveolae was described in Cav-3 KO mice although Cav-1 levels remained stable (Hagiwara et al., 2000; Horikawa et al., 2008; Tsutsumi et al., 2010b). Furthermore, these mice lose the ability to undergo preconditioning-like cardiac protection from ischemia/reperfusion injury (Horikawa et al., 2008). These results implicate a role for Cav-3 and the presence of caveolae in cardiac protection from ischemia/reperfusion injury. The current data show that GGA similarly requires Cav-3 and caveolae. These effects are likely due to the ability of Cav-3 and caveolae to be able to regulate mitochondrial pore activity and mediate mitochondrial mediated cell death. Furthermore, recent investigations have revealed that caveolae and caveolins can directly interact with mitochondria and regulate mitochondrial function, suggesting a potential regulatory role in surface and inter-organellar signaling (Fridolfsson et al., 2012).

In the present study, we show that the administration of GGA led to a significant increase in Cav-3 protein expression within the buoyant fraction, and that HSP 70 directly localized and interacted with Cav-3. Many previous studies using GGA have focused on HSP 70 induction (Hirakawa et al., 1996; Sakabe et al., 2008) and have suggested that GGA protects the myocardium by activating heat shock proteins especially HSP 72 (Kitahata et al., 2008; Ooie et al., 2005; Ooie et al., 2001; Yamanaka et al., 2003). Although there has been little evidence regarding the relationship between caveolae, caveolins and heat shock proteins within the heart, other organ systems including skin and cancer have investigated this relationship. In skin models, HSP have been shown to localize into caveolae, which then can regulate intracellular HSP expression (Black et al., 2011). Furthermore, in tumor cells, Cav-1 has been shown to have direct effects on HSP expression (Ciocca et al., 2012). Perhaps, in regard to GGA, caveolae are spatial hubs that localize GGA and HSP, together regulating temporal relations and activation. Given the multifaceted nature of caveolae and caveolins, multiple survival pathways are being activated simultaneously.

Glucose transporter 4 receptor (Glut4) has also been implicated in delayed preconditioning of the myocardium. Interestingly, Cav-3 KO mice are unable to be protected via isoflurane, due to its inability to translocate Glut 4, although this is preserved in Cav-1 KO, making this a Cav-3 specific phenomenon (Tsutsumi et al., 2010b). Perhaps GGA may also have effects on Glut4 translocation and expression.

There are several limitations in the present study. First, we evaluated the effects of Cav-3 and HSP 70 in experiments that investigated ischemia/reperfusion injury. GGA induces a broad class of the protective proteins, including various families of HSPs (Brundel et al., 2006a, 2006b), protein kinase C (Yamanaka et al., 2003), and nitric oxide (Yamamoto et al., 2005). Second, the dose and time period used for cardiac protection in mice are unknown. Previous studies have all been in rats, where a single oral dose of 200 mg/kg GGA induced HSP and cardiac protection against ischemia/reperfusion (Ooie et al., 2005, 2001;

Shinohara et al., 2007; Yamanaka et al., 2003). The same concentration and dose were applied in this study.

Taken together these data demonstrate that GGA had delayed cardio-protective effects that are dependent on caveolae and Cav-3. We show that caveolae and Cav-3 are the vital conduits between HSP-70 activation and subsequent mitochondrial respiration suggesting an important role for this structural protein. Furthermore, these data suggest that GGA may be a unique way to clinically increase caveolae and caveolin-3 expression, which have both shown to have significant cardioprotective effects against ischemic injury and detrimental remodeling.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

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# *Epac1*-dependent phospholamban phosphorylation mediates the cardiac response to stresses

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PKA phosphorylates multiple molecules involved in calcium ( $\text{Ca}^{2+}$ ) handling in cardiac myocytes and is considered to be the predominant regulator of  $\beta$ -adrenergic receptor-mediated enhancement of cardiac contractility; however, recent identification of exchange protein activated by cAMP (EPAC), which is independently activated by cAMP, has challenged this paradigm. Mice lacking *Epac1* (*Epac1* KO) exhibited decreased cardiac contractility with reduced phospholamban (PLN) phosphorylation at serine-16, the major PKA-mediated phosphorylation site. In *Epac1* KO mice, intracellular  $\text{Ca}^{2+}$  storage and the magnitude of  $\text{Ca}^{2+}$  movement were decreased; however, PKA expression remained unchanged, and activation of PKA with isoproterenol improved cardiac contractility. In contrast, direct activation of EPAC in cardiomyocytes led to increased PLN phosphorylation at serine-16, which was dependent on PLC and PKC $\epsilon$ . Importantly, *Epac1* deletion protected the heart from various stresses, while *Epac2* deletion was not protective. Compared with WT mice, aortic banding induced a similar degree of cardiac hypertrophy in *Epac1* KO; however, lack of *Epac1* prevented subsequent cardiac dysfunction as a result of decreased cardiac myocyte apoptosis and fibrosis. Similarly, *Epac1* KO animals showed resistance to isoproterenol- and aging-induced cardiomyopathy and attenuation of arrhythmogenic activity. These data support *Epac1* as an important regulator of PKA-independent PLN phosphorylation and indicate that *Epac1* regulates cardiac responsiveness to various stresses.

## Introduction

$\beta$ -Adrenergic receptor ( $\beta$ -AR) signaling is well established as a primary defense mechanism against acute stress or changes in hemodynamic load; however, its role in cardiac pathogenesis, although studied extensively, remains poorly understood (1).  $\beta$ -AR blockade, rather than stimulation, is beneficial in patients with heart failure, and pharmacological inhibition of the entire  $\beta$ -AR signaling pathway at the receptor level is one of the accepted treatments for heart failure (2). Studies in mouse models overexpressing  $\beta$ -AR, Gs $\alpha$ , or PKA support the usefulness of  $\beta$ -AR blockade in heart failure (3–5). Recently, however, we have developed a mouse model in which type 5 adenylyl cyclase (AC), a major cardiac subtype, is disrupted (*Ac5* KO), and we found that *Ac5* KO showed resistance to the development of heart failure and exhibited increased longevity (6–9), indicating that inhibition of cAMP signaling at the level of AC, not  $\beta$ -AR, may also result in cardiac protection.

Systolic contraction follows activation of sarcolemmal voltage-gated L-type calcium ( $\text{Ca}^{2+}$ ) channel during an action potential, resulting in  $\text{Ca}^{2+}$ -influx, which activates cardiac ryanodine receptor (RyR2)  $\text{Ca}^{2+}$  release channels on the sarcoplasmic reticulum (SR) (major intracellular  $\text{Ca}^{2+}$  store). Diastolic relaxation occurs with cessation of  $\text{Ca}^{2+}$  release, and  $\text{Ca}^{2+}$  sequestration by the SR  $\text{Ca}^{2+}$ -transporting adenosine triphosphatase (SERCA2a) and its regulator, phospholamban (PLN), indicate that phosphorylations of PLN and RyR2 have a central role in modulating  $\text{Ca}^{2+}$  homeostasis and, therefore, cardiac function (10, 11). PLN is a low-molecular weight phosphoprotein in cardiac SR, and dephosphorylated PLN is an inhibitor of SERCA2a-mediated transport of  $\text{Ca}^{2+}$ . Following  $\beta$ -AR activation, and thus production of cAMP by AC, PKA phosphorylates PLN on serine-16 (10) in addition to RyR2 on serine-2808 and serine-2814 (11). PKA-mediated serine-16 phosphorylation (and thus SERCA2a activation) is the major mechanism of the lusitropic and also inotropic (12) effects of  $\beta$ -AR stimulation in regulating cardiac function (13). In failing hearts, in contrast, decreased PLN phosphorylation, and thus a decrease in  $\text{Ca}^{2+}$  uptake by SERCA2a, is a central feature (14, 15). Indeed, decreased inhibition of SERCA2a by PLN ablation can prevent progression of heart failure (16, 17).

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## research article

**Table 1**  
Heart size and cardiac function in *Epac1* KO

	WT (n)	<i>Epac1</i> KO (n)
Age, month	4.2 ± 0.1 (32)	4.2 ± 0.2 (21)
BW, g	30 ± 0.7 (21)	29 ± 0.9 (18)
Tibia, mm	17.5 ± 0.1 (21)	17.4 ± 0.1 (18)
LV/tibia (mg/mm)	5.2 ± 0.2 (20)	5.2 ± 0.2 (18)
LV/BW (mg/g)	3.2 ± 0.1 (20)	3.3 ± 0.2 (18)
LVEDD, mm	4.3 ± 0.03 (31)	4.4 ± 0.1 (18)
LVESD, mm	2.9 ± 0.04(31)	3.3 ± 0.1 (18) <sup>A</sup>
LVEF, %	70 ± 0.8 (31)	60 ± 1.1 (18) <sup>A</sup>
%FS	35 ± 1.1 (31)	26 ± 0.6 (18) <sup>A</sup>
Max dP/dt, mmHg	9091 ± 240 (17)	7246 ± 226 (16) <sup>A</sup>
Min dP/dt, mmHg	-9110 ± 240 (18)	-6201 ± 125 (14) <sup>A</sup>

Data are mean ± SEM. LVEDD, LV end-diastolic diameter. FS, fractional shortening. <sup>A</sup>*P* < 0.01.

Others have found that decreased inhibition of SERCA2a through PLN ablation or hyperphosphorylation of PLN exaggerated heart failure and arrhythmogenic activity (18–22). Thus, it remains controversial whether or not enhancement of Ca<sup>2+</sup> uptake through PLN ablation or PLN hyperphosphorylation can inhibit the progression of heart failure. Nevertheless, it is well accepted that β-AR-mediated phosphorylation of PLN plays a central role in regulating cardiac function and also in the pathogenesis of cardiac failure.

But PKA is not the only molecule activated by cAMP. Exchange protein activated by cAMP (EPAC) was recently identified as a new target of cAMP signaling that is activated independently of PKA (23, 24). EPAC has 2 isoforms (*Epac1* and *Epac2*), and *Epac1* is predominantly expressed in the heart. EPAC modulates various cellular functions, including migration, proliferation, exocytosis, and apoptosis, via regulation of RAP1 (25). In cardiac myocytes, EPAC activation induced hypertrophy (26). In adult mouse cardiac myocytes, pharmacological EPAC activation with an EPAC-selective but not isoform-selective cAMP analogue was reported to enhance evoked Ca<sup>2+</sup> transients through the PLCε/PKCε/CaMKII pathway, and activation of CaMKII via EPAC induced store depletion and enhancement of Ca<sup>2+</sup> sparks through increased phosphorylation of PLN on threonine-17 and RyR2 on serine-2815 by EPAC via phospholipase C (PLC) ε/PKC ε/Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) (27–29). EPAC2 was also reported to be closely associated with CaMKII, and EPAC2 mediated β<sub>1</sub>-AR-induced cardiac arrhythmia via CaMKIIδ (a major isoform of cardiac CaMKII) and RyR2 phosphorylation on serine-2815 (30). However, the isoform-specific role of EPAC in cardiac function and pathogenesis remains poorly understood.

Here, we show that EPAC1, in an additive and independent manner with respect to PKA, phosphorylates PLN and RyR2 to regulate cardiac function. Loss of EPAC1 slightly decreased basal cardiac function, but afforded greater protection against various stresses, including arrhythmogenic stress, whereas loss of EPAC2 did not show cardioprotective effects. Accordingly, selective EPAC1 inhibition may prevent hyperphosphorylation of PLN and RyR2, and this may be an alternative strategy to current β-AR blocker therapy for the treatment of established cardiac failure or arrhythmia.

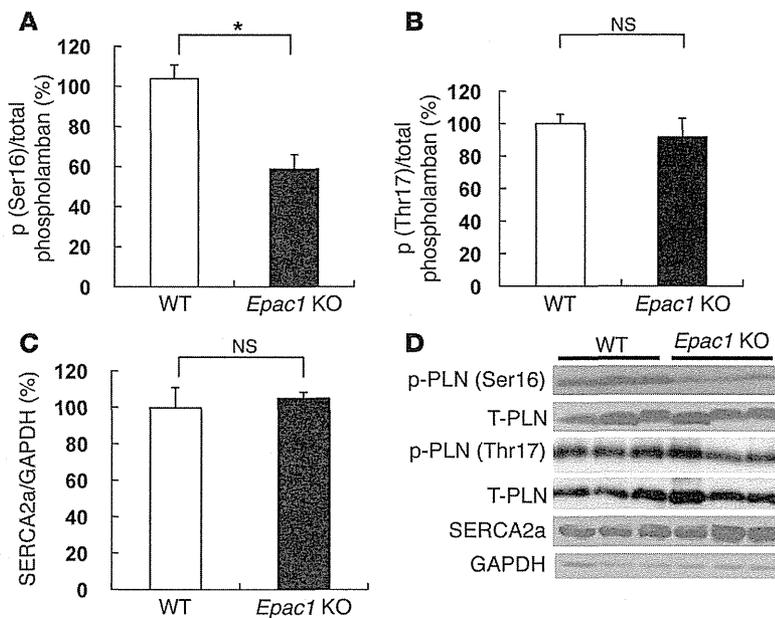
## Results

*Cardiac function is decreased in Epac1 KO.* Because EPAC activation induced cardiac myocyte hypertrophy in culture (26), we originally

thought that EPAC might play a role in regulating cardiac myocyte growth. Therefore, we genetically disrupted *Epac1* in mice (*Epac1* KO) (31), but found that these mice did not show any change in the size of the heart. Instead, *Epac1* KO exhibited a modest, but significant, decrease in cardiac function. LV ejection fraction (LVEF) was significantly decreased (WT versus *Epac1* KO: 70% ± 0.8% versus 60% ± 1.1%, *n* = 18–31, *P* < 0.01), and LV end-systolic diameter (LVESD) was increased (WT versus *Epac1* KO: 4.3 ± 0.03 versus 4.4 ± 0.1 mm, *n* = 18–31, *P* < 0.01) (Table 1). Hemodynamic measurements demonstrated that Max dP/dt was significantly decreased (WT versus *Epac1* KO: 9091 ± 240 versus 7246 ± 226 mmHg, *n* = 16–17, *P* < 0.01), and Min dP/dt was significantly increased in *Epac1* KO (WT versus *Epac1* KO: -9110 ± 240 versus -6201 ± 125 mmHg, *n* = 14–18, *P* < 0.01) (Table 1). Because disruption of *Epac1* might alter the expression of other molecules involved in cAMP signaling, such as PKA subunits, we examined the protein expression of β<sub>1</sub>-AR, β<sub>2</sub>-AR, β-AR kinase (βARK), Gsα, Giα, Gβ, Gγ, and type 5/6 AC in addition to PKA subunits by means of Western blotting (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI64784DS1). There was no difference in expression of these molecules between WT and *Epac1* KO. Also, there was no compensatory increase of *Epac2* isoform in *Epac1* KO at either the mRNA or protein level (Supplemental Figure 1, A and B).

Decreased cardiac function in *Epac1* KO was readily compensated by isoproterenol (ISO) infusion. Responses of LVEF and LVESD to ISO (0, 0.13, 0.27, 0.40 μg/kg/min for 5 minutes) were well preserved and, indeed, reached the same level as those of WT (Supplemental Figure 2, A and B). Changes in cardiac contractility were further examined using isolated segments of myocardium (Supplemental Figure 3). The basal contractile force was significantly smaller in *Epac1* KO (WT versus *Epac1* KO: 112 ± 14.7 versus 73 ± 7.1 mg/mm<sup>2</sup>, *n* = 6–7, *P* < 0.05) (Supplemental Figure 3A). ISO dose dependently enhanced the contractile force in WT and *Epac1* KO. The magnitude of increase in response to ISO was similar in WT and *Epac1* KO (Supplemental Figure 3B). The sensitivity to β-adrenergic stimulation with ISO as represented by the -log EC<sub>50</sub> value for the enhancement of contractile force was 7.04 ± 0.07 in WT and 7.06 ± 0.01 in *Epac1* KO (*P* = NS, *n* = 6–7). The time required to reach peak tension was also similar in *Epac1* KO and WT at baseline (WT versus *Epac1* KO: 50 ± 1.1 versus 52 ± 1.5 ms, *n* = 6–7, *P* = NS) and decreased similarly in both WT and *Epac1* KO (Supplemental Figure 3C). However, the time required for 90% relaxation was significantly longer in *Epac1* KO at baseline (WT versus *Epac1* KO: 54 ± 1.5 versus 61 ± 2.0 ms, *n* = 6–7, *P* < 0.05), but became similar in response to ISO (Supplemental Figure 3D). Thus, *Epac1* KO showed decreased cardiac function, exemplified most clearly by delayed relaxation, but the dysfunction was readily compensated by β-AR stimulation.

*PLN phosphorylation on serine-16 is decreased in Epac1 KO.* Phosphorylation of PLN occurs, following β-adrenergic stimulation, on serine-16 and threonine-17. Indeed, PKA-mediated serine-16 phosphorylation is a key event in increasing cardiac function (32). Unexpectedly, phosphorylation on serine-16 was significantly decreased in *Epac1* KO (WT versus *Epac1* KO: 100% ± 6.5% versus 57% ± 7.1%, *n* = 6, *P* < 0.01) (Figure 1A) despite the absence of any difference in expression of the PKA catalytic and regulatory units (RIα, RIIα) (Supplemental Figure 1A). PLN phosphorylation on threonine-17 (Figure 1B), which is mediated by CaMKII, was similar in WT and *Epac1* KO. Phosphorylation on threonine-286, and thus activation of CaMKII, was also similar (Supplemental Figure 4).

**Figure 1**

Phosphorylation of PLN on serine-16 and threonine-17 and SERCA2a expression in the heart of *Epac1* KO at baseline. **(A)** Phosphorylation on serine-16 (Ser16) was significantly decreased in *Epac1* KO compared with WT (WT versus *Epac1* KO:  $100\% \pm 6.5\%$  versus  $57\% \pm 7.1\%$ ,  $n = 6$ ,  $*P < 0.01$ ). The ratio of phosphorylated/total protein expression of PLN in WT was taken as 100% in each determination. **(B)** Phosphorylation on threonine-17 (Thr17) was not different in *Epac1* KO and WT (WT versus *Epac1* KO:  $100\% \pm 5.7\%$  versus  $92\% \pm 11.5\%$ ,  $n = 7-10$ ,  $P = NS$ ). The ratio of phosphorylated/total protein expression of PLN in WT was taken as 100% in each determination. **(C)** The expression of SERCA2a protein was not different in WT and *Epac1* KO at baseline (WT versus *Epac1* KO:  $100\% \pm 11.1\%$  versus  $105\% \pm 3.5\%$ ,  $n = 6-7$ ,  $P = NS$ ). The ratio of SERCA2a protein/GAPDH in WT was taken as 100% in each determination. **(D)** Representative immunoblotting results of phosphorylation of PLN on serine-16 (upper) and threonine-17 (middle) and SERCA2a (lower) are shown. T-PLN, total PLN; p-PLN, phosphorylated PLN.

*EPAC-selective cAMP analogue increases PLN phosphorylation on serine-16 and threonine-17 in neonatal rat cardiac myocytes.* The above findings indicated that disruption of EPAC, but not PKA, decreased PLN phosphorylation. When we examined the consequences of EPAC activation for PLN phosphorylation in neonatal rat cardiac myocytes, we used a new membrane-permeable EPAC-selective but not isoform-selective agonist, i.e., 8-(4-chlorophenylthio)-2'-O-Me-cAMP-AM (8-CPT-AM) (33). We found that  $10 \mu\text{M}$  8-CPT-AM was necessary to obtain a significant PLN phosphorylation signal with our detection system in neonatal rat cardiac myocytes (Figure 2D), and thus this concentration was used. This concentration of 8-CPT-AM is in line with those used in previous studies to examine EPAC-mediated signaling, i.e.,  $10 \mu\text{M}$  for adult rat cardiac myocytes (34) or  $50 \mu\text{M}$  for 293T, JAR, and BeWo cells (35). Furthermore, Brette et al. reported very recently that 8-CPT-AM at  $10 \mu\text{M}$  did not exert an inhibitory effect on phosphodiesterase (PDE) activity (34). On the other hand, non-AM ester 8-CPT (8-CPT) does inhibit PDE, which is undesirable, since we wish to examine EPAC-specific effects (36, 37).

We found that 8-CPT-AM significantly increased the PLN phosphorylation on serine-16 by approximately 89-fold from baseline and that on threonine-17 by approximately 19-fold from baseline at 15 minutes after the treatment with 8-CPT-AM ( $n = 4$ ,  $P < 0.01$ ) (Figure 2, A and B). PLN phosphorylation on serine-16 was decreased gradually, but was significantly increased even at 120 minutes after 8-CPT-AM treatment. However, PLN phosphorylation on threonine-17 decreased rapidly and did not show a significant increase at 60 minutes after 8-CPT-AM treatment. Thus, activation of EPAC with 8-CPT-AM, in addition to PKA and CaMKII, could induce PLN phosphorylation on serine-16 and threonine-17, but PLN phosphorylation on serine-16 persists for a long time, compared with that on threonine-17, which has been reported to be phosphorylated by EPAC via PLC $\epsilon$ /PKC $\epsilon$ /CaMKII (27, 28).

*Increase of PLN phosphorylation at serine-16 was attenuated in neonatal cardiac myocytes prepared from Epac1 KO after the treatment of 8-CPT-AM.* We examined the effects of 8-CPT-AM on PLN

phosphorylation at serine-16 using neonatal cardiac myocytes prepared from *Epac1* KO and WT. In WT neonatal mouse cardiac myocytes, we found that  $1 \mu\text{M}$  8-CPT-AM was sufficient to obtain a significant PLN phosphorylation signal with our detection system (Supplemental Figure 5). Since the magnitude of the increase was similar to that obtained with  $10 \mu\text{M}$  8-CPT-AM in neonatal rat cardiac myocytes, we used this concentration ( $1 \mu\text{M}$ ) (Figure 2D and Supplemental Figure 5). EPAC activation with 8-CPT-AM ( $1 \mu\text{M}$ ) significantly increased PLN phosphorylation at serine-16 from baseline in WT, and the level was also significantly greater than that in *Epac1* KO ( $P < 0.05$ ). In contrast with WT, the increase of PLN phosphorylation at serine-16 was not significant (WT: from  $100\% \pm 11\%$  to  $1534\% \pm 528\%$ ,  $n = 4-6$ ,  $P < 0.01$ ; *Epac1* KO: from  $61\% \pm 22\%$  to  $476\% \pm 141\%$ ,  $n = 4-6$ ,  $P = NS$ ). These results, together with the data shown in Figure 1A and Figure 2A, indicate that EPAC1 is an important regulator of PLN phosphorylation at serine-16, independently of PKA. More importantly, these findings also suggest that 8-CPT-AM at the concentration of  $1 \mu\text{M}$  phosphorylates PLN at serine-16 via EPAC, not other signaling pathways, such as PKA, cGMP-dependent PKG, or PDE (34, 38).

*EPAC phosphorylates PLN at serine-16 via PLC/PKC $\epsilon$ .* It is established that PKC phosphorylates PLN and that the phosphorylation via PKC is additive to that via PKA and CaMKII, but the significance of these findings and the mechanisms involved remain poorly understood (39-41). It has been suggested that EPAC mediates a novel pathway of regulatory crosstalk between the cAMP and the PLC/PKC pathways (42), and so we hypothesized that EPAC mediates PLN phosphorylation on serine-16 via PLC/PKC. Indeed, in the presence of PLC inhibitor (U73122) or PKC inhibitor (Ro-31-7549), EPAC-mediated phosphorylation of serine-16 was negated in neonatal mouse and rat cardiac myocytes (Supplemental Figure 6). Twelve PKC isoforms are known to exist; conventional PKC subtypes, which include PKC $\alpha$ , PKC $\beta$ , and PKC $\gamma$ , require both  $\text{Ca}^{2+}$  and phospholipid for activation, whereas novel PKC subtypes, which include PKC $\delta$  and PKC $\epsilon$ , require phospholipid, but not  $\text{Ca}^{2+}$  (43). We examined the effect of silencing each