Molecular Screen Identifies Cardiac Myosin–Binding Protein-C as a Protein Kinase G-Iα Substrate

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Background—Pharmacological activation of cGMP-dependent protein kinase G I (PKG) has emerged as a therapeutic strategy for humans with heart failure. However, PKG-activating drugs have been limited by hypotension arising from PKG-induced vasodilation. PKG-Iα antiremodeling substrates specific to the myocardium might provide targets to circumvent this limitation, but currently remain poorly understood.

Methods and Results—We performed a screen for myocardial proteins interacting with the PKGIα leucine zipper (LZ)–binding domain to identify myocardial-specific PKGI antiremodeling substrates. Our screen identified cardiac myosin–binding protein-C (cMyBP-C), a cardiac myocyte–specific protein, which has been demonstrated to inhibit cardiac remodeling in the phosphorylated state, and when mutated leads to hypertrophic cardiomyopathy in humans. GST pulldowns and precipitations with cGMP-conjugated beads confirmed the PKGIα–cMyBP-C interaction in myocardial lysates. In vitro studies demonstrated that purified PKG-Iα phosphorylates the cMyBP-C M-domain at Ser-273, Ser-282, and Ser-302. cGMP induced cMyBP-C phosphorylation at these residues in COS cells transfected with PKGIα, but not in cells transfected with LZ mutant PKGIα, containing mutations to disrupt LZ substrate binding. In mice subjected to left ventricular pressure overload, PKG activation with sildenafil increased cMyBP-C phosphorylation at Ser-273 compared with untreated mice. cGMP also induced cMyBP-C phosphorylation in isolated cardiac myocytes.

Conclusions—Taken together, these data support that PKGIα and cMyBP-C interact in the heart and that cMyBP-C is an antiremodeling PKGIα kinase substrate. This study provides the first identification of a myocardial-specific PKGIα LZ-dependent antiremodeling substrate and supports further exploration of PKGIα myocardial LZ substrates as potential therapeutic targets for heart failure. (Circ Heart Fail. 2015;8:1115-1122. DOI: 10.1161/CIRCHEARTFAILURE.115.002308.)

Key Words: cyclic GMP-dependent protein kinase type I ■ heart failure ■ leucine zippers ■ myosin-binding protein C ■ ventricular remodeling

The cGMP-dependent protein kinase G I (PKG) regulates functions in multiple cardiovascular tissues, including the systemic vasculature, where it induces vasorelaxation, and the myocardium,1 where its role remains less completely understood. Preclinical studies have revealed that PKG signaling inhibits the process of pathological cardiac remodeling in vivo.2-5 Pharmacological strategies to activate PKGI, including phosphodiesterase inhibition (sildenafil),6-7 natriuretic peptide administration (nesiritide and carperitide),8,9 and nitrate therapy,10 have emerged as potential antiremodeling treatments for patients with heart failure (HF).1 Recently, the Prospective Comparison of ARNI with ACEI to Determine Impact on Global Mortality and Morbidity in Heart Failure (PARADIGM-HF) trial of the combined angiotensin receptor–neprilysin inhibitor LCZ696 demonstrated reduced death from cardiovascular causes or first HF hospitalization in patients with HF with reduced ejection fraction.11 LCZ696 activates PKGI by preventing neprilysin enzymatic breakdown of extracellular natriuretic peptides, further supporting the clinical efficacy of PKG activation in HF.11

Clinical Perspective on p 1122

Importantly, LCZ696 and other pharmacological PKGI activators have been limited to date by significant hypotension arising from PKG-I-induced vasodilation.1,11 Identifying downstream PKGI substrates specific to the myocardium might therefore reveal targets to inhibit remodeling yet avoid excessive hypotension. However, PKGI antiremodeling substrates in the myocardium remain poorly understood. PKGIα, the predominant PKGI isoform in the heart, contains a unique N-terminal leucine zipper (LZ) domain which mediates binding with critical kinase substrates.12 Global mutation of
the PKG1α LZ domain leads to accelerated HF and cardiac remodeling in response to LV pressure overload by trans-aortic constriction (TAC), supporting an important role of PKG1α LZ substrates in attenuating pathological remodeling. However, these PKG1α LZ–binding substrates specific to the myocardium have not been explored.

We therefore undertook the present study to identify PKG1α LZ–binding partners as candidate antiremodeling substrates in the myocardium. To this end, we designed a molecular screen for myocardial PKG1α LZ–binding molecules. Here, we describe the identification of cardiac myosin–binding protein-C (cMyBP-C), a CM-specific protein and critical inhibitor of cardiac remodeling, as a PKG1α LZ–binding partner and kinase substrate.

Methods

Preparation of GST Fusion Proteins

pGEX plasmids containing the N-terminal amino acids 1 to 59 of the wild-type (WT) PKG1α LZ domain (PKG1α–59), mutated LZ domain (PKG1α–59-M), and glutathione S-transferase (GST) alone were transformed into BL21, as described.12 Bacteria were treated with isopropyl beta-D-thiogalactopyranoside (0.2 mmol/L×2.5 hours at 30°C) to induce protein translation, followed by generation of GST fusion beads as described.12

Myocardial Screen

Hearts of C57/Bl6 male mice were rapidly excised and snap frozen in liquid nitrogen, followed by dounce homogenization of the left ventricle (LV) in lysis buffer as described previously.1 LV lysates were rocked gently overnight at 4°C with fusion protein beads, followed by washing 3 times with lysis buffer. Proteins were eluted off the beads by boiling for 5 minutes in Laemmli sample buffer, followed by separation by SDS-PAGE and Coomassie staining. Bands, which were identified by mass spectroscopy, were analyzed by mass spectrometry at the Harvard Taplin Mass Spectroscopy facility as described.13

Western Blotting

GST pulldowns and PKG1α precipitation with cGMP and RP-8AET-GST were performed as described.14 Western blotting for PKG1α and cMyBP-C was performed with anti-PKG1α14 and anti-cMyBP-C (Santa Cruz) antibodies at 1:1000 and 1:100, respectively. For in vivo phosphorylation studies, antibodies to cMyBP-C, and antiphospho-specific antibodies against Ser-273, Ser-282, and Ser-302.15 The C0-C1-M-C2 domain was used as described in the manufacturer protocol.

Cardiac Myocyte Culture

Adult rat ventricular myocytes were isolated as previously described16 with the following modifications. Adult rat ventricular myocytes were obtained from adult male Sprague–Dawley rats (Harlan Laboratories, 175–200 g) and were plated at a nonconfluent density (25–50 cells/mm²) on laminin-coated dishes in DMEM low-glucose media (Invitrogen, 12320-032) supplemented with 0.1% penicillin–streptomycin. After 1 hour of plating, the media were replaced, and the cells were used for experimentation the following day as indicated in the figure legends.

Animal Models

All studies were performed under institutional approval by the Tufts Institutional Animal Care and Use Committee. TAC was performed as described17 for a duration of 5 weeks. Sildenafil was administered orally at a dose of 200 mg/kg per day as described,17 beginning 1 week after TAC.

Cotransfection Experiments

CV-1 in Origin, carrying the SV40 genetic material (COS) cells were transfected with full-length cMyBP-C, a plasmid containing the human cytomegalovirus major intermediate-early gene enhancer region (termed the pCI vector), and with either WT or LZM PKG1α in pCI. Cells were treated with 100 μmol/L 8Br-cGMP for 10 minutes and with 20 mmol/L KT 5720, a selective cAMP-dependent protein kinase A inhibitor, to prevent potential nonspecific cGMP-induced protein kinase A activation. Cells were lysed as described,17 and samples subjected to SDS-PAGE and Western blot.

In Vitro Phosphorylation of cMyBP-C by PKG1α

Recombinant mouse His-tagged cMyBP-C spanning the C0-C1-M-C2 domains was generated in Escherichia Coli using a plasmid expression vector driven by the T7 promoter (pET expression system). Recombinant PKG1α (4 U; Cat no. CY-E1161-2, MBL International Corporation, Woburn, MA) was incubated with 100 ng of His-tagged cMyBP-C for 30 minutes at 37°C. Reactions were incubated with either vehicle or with 100 μmol/L 8Br-cGMP. Reactions were stopped with 2x loading dye and heat denaturation. Proteins were resolved on 4% to 16% SDS-PAGE gels, followed by Western blot analyses against anti-C0 domain–specific antibodies for cMyBP-C, and antiphospho-specific antibodies against Ser-273, Ser-282, and Ser-302.15 The C0-C1-M-C2 domain was also detected by silver stain. In the phosphorylation assay, a specific PKG1α inhibitor Rp-8-pCPT-cGMP (MBL International Corporation, Woburn, MA) was used as described in the manufacturer protocol.

Figure 1. Screening strategy for identifying protein kinase G Iα (PKG1α) leucine zipper (LZ) interacting proteins in the myocardium. A, Outline of screening strategy. GST fusion proteins were generated containing the PKG1α LZ domain (PKG1α–59), the PKG1α–mutated LZ domain (PKG1α–59-M), or GST alone. The separate bait proteins were incubated with left ventricular protein lysates, followed by SDS-PAGE and Coomassie staining. Protein bands selectively precipitating with PKG1α–59 were removed and identified by mass spectroscopy. B, Representative Coomassie stain from left ventricular protein lysates incubated with GST fusion proteins. The 150-kDa band visible only in PKG1α–59 precipitate (denoted by arrow) was excised and subjected to mass spectrometry, revealing cardiac myosin–binding protein-C (cMyBP-C) as the predominant species. The thick bands between 25 and 30 kDa represent GST fusion proteins. Representative of 3 independent experiments. LV indicates left ventricle.

Statistical Analysis

Quantitative data are presented as dot plots with the sample mean denoted by a horizontal bar. Two-sample comparisons were analyzed by the Mann–Whitney rank sum test. Multiple groups were compared...
using Kruskal–Wallis 1-way ANOVA on ranks, followed by multiple comparison testing with the Tukey method (or Dunn method if group sizes differed). Multiple comparisons were performed if the initial Kruskal–Wallis test was significant or near significant, as described in the figure legends. Data were analyzed with SigmaPlot version 12.5.

**Results**

**Identification of Cardiac Myosin Protein-C Through a Screen for PKGI α LZ Interacting Proteins**

To screen for specific PKGI α LZ interacting proteins in the myocardium, we incubated LV lysates with GST fused to: the WT PKGI α LZ domain (PKG1–59); the PKGI α LZ mutant domain (PKG α LZM); or with GST alone, followed by separation by SDS-PAGE and Coomassie staining. Figure 1A outlines the screening strategy. The Coomassie stain identified a band at ≈150 kDa, detected only in the PKG1–59 pulldowns (Figure 1). Mass spectroscopy revealed cMyBP-C as the predominant protein in the band. cMyBP-C is a CM-specific protein, which is required for normal CM and LV diastolic and systolic function, although its regulation by PKGI α has not been described. Of note, in other PKG1–59-specific bands, we also detected proteins previously shown to bind the PKGI α LZ domain, including tropinin I α and PKGI α, which is known to form a homodimer through LZ–LZ interactions. We therefore explored this potential PKGI α–cMyBP-C binding further. To confirm that the PKGI α LZ domain precipitates cMyBP-C, we performed, on the same GST pulldown samples, Western blotting for cMyBP-C. cMyBP-C was detected with the PKG1–59, α WT pulldown, but not PKG α LZM or GST alone (Figure 2A), supporting a specific interaction with the WT LZ domain. We next tested whether full-length PKGI α also complexes with cMyBP-C in the LV. We incubated LV lysates with agarose-conjugated cGMP and RP-8AET-cGMP beads, which each bind PKGI with high affinity and activate or inhibit PKGI kinase activity, respectively. Precipitation of PKGI α with either of the cGMP-linked beads, but not with control agarose beads, coprecipitated cMyBP-C (Figure 2B), supporting cointeraction of native PKGI α and cMyBP-C in the myocardium.

**Requirement of PKGI α LZ Domain for Phosphorylation of Full-Length cMyBP-C**

cMyBP-C contains multiple phosphorylation sites, including 3 serine residues on the M-domain (Ser-273, Ser-282, and Ser-302). Prior studies identified that phosphorylation of cMyBP-C on these 3 M-domain sites by protein kinase A improves cardiac myocyte (CM) and LV systolic and diastolic function. In addition, phosphorylation status of these sites inhibits pathological remodeling in vivo. Group-based phosphorylation scoring analysis of cMyBP-C strongly predicted PKGI phosphorylation at each of these 3 sites (Figure 3A). We therefore tested the requirement of the PKGI α LZ domain for the phosphorylation of full-length cMyBP-C. We cotransfected COS cells with full-length cMyBP-C and either WT PKGI α or LZ mutant PKGI α. The membrane-permeable cGMP analogue 8Br-cGMP (100 μM/L) induced cMyBP-C phosphorylation at Ser-273, Ser-282, and Ser-302 in WT PKGI α-transfected cells (fold increase p/ control: 1.63±0.19, 1.66±0.02, and 1.92±0.19 arbitrary densitometric units (ADUs) in serine 273, 282, and 302, respectively). However, 8Br-cGMP failed to induce phosphorylation at these residues in the cells transfected with LZ mutant PKGI α (Figure 3). These findings support that cGMP induces PKGI α phosphorylation of cMyBP-C, and moreover that the PKGI α LZ domain is required for this cGMP-induced phosphorylation.

**Direct PKGI α Phosphorylation of cMyBP-C**

We next tested whether PKGI α directly phosphorylates cMyBP-C in vitro, by incubating purified PKGI α with recombinant His-tagged cMyBP-C spanning the C0-C1-M-C2 domains (Figure 4A). PKGI α induced cMyBP-C phosphorylation of: 10.42±0.81 ADUs on Ser-273; 2.37±0.63 ADUs on Ser-282; and 2.34±0.14 ADUs on Ser-302. PKGI α activation with the cGMP analogue 8Br-cGMP (100 μM/L) further increased cMyBP-C phosphorylation at these sites to 20.34±3.5 ADUs on Ser-273 (P<0.05 versus control); 4.50±1.02 ADUs on Ser-282 (P<0.05 versus control); and 3.07±0.42 ADUs on Ser-302 (P<0.05 versus control; Figure 4). Furthermore, the PKGI α-specific inhibitor Rp-8CPT-cGMP decreased PKGI α-induced phosphorylation of these 3 sites (Figure 4), supporting the specificity of this reaction.

**In Vivo Regulation of cMyBP-C by PKGI α in the Pressure Overloaded LV**

The phosphodiesterase 5 inhibitor, sildenafil, activates PKG in the myocardium and inhibits pressure overload–induced remodeling and HF. We tested whether PKGI α activation with sildenafil increased cMyBP-C phosphorylation in mice subjected to LV pressure overload by TAC for 5 weeks. In these previously reported mice, sildenafil improved LV fractional shortening and reduced LV mass compared with vehicle-treated mice, as well as increasing PKGI kinase activity. Using cardiac tissues from these previously reported mice, we measured the phosphorylation state of cMyBP-C. Compared with vehicle-treated mice, sildenafil treatment induced a 2.5±0.26-fold increase in cMyBP-C phosphorylation of...
Ser-273 in mice subjected to TAC (P < 0.05; Figure 5). Sildenafil treatment did not affect cMyBP-C phosphorylation at Ser-282 and Ser-302 (data not shown). These findings indicate that pharmacological activation of PKGI induces myocardial phosphorylation of cMyBP-C at Ser 273 in the setting of pressure overload and suggests that this may mediate the antiremodeling effect of PKGI in this model.

To examine whether cGMP augmentation directly regulated cMyBP-C phosphorylation in the CM, as opposed to a secondary effect, we tested the effect of 8-Br-cGMP on cMyBP-C phosphorylation in isolated adult rat ventricular myocytes. 8-Br-cGMP induced a dose-dependent increase in cMyBP-C phosphorylation at Ser-273 and Ser-282 compared with vehicle-treated CMs (100 μmol/L 8-Br-cGMP induced 44.1 ± 7.8-fold increase on Ser-273, P < 0.05 versus vehicle; and 5.7 ± 0.6-fold increase on Ser-282, P < 0.05 versus vehicle; Figure 5C and 5D).

Discussion

In this study, we performed a molecular screen for myocardial PKGα substrates, based on the growing clinical development of PKG activation strategies to treat HF. We observed (1) selective precipitation of cMyBP-C with the PKGα LZ–binding domain in LV lysates; (2) cointeraction of PKGα and cMyBP-C in the heart; (3) phosphorylation of cMyBP-C by PKGα in vitro and in cell culture in an LZ-dependent fashion, on sites previously shown to inhibit remodeling when phosphorylated; and (4) increased cMyBP-C phosphorylation on Ser-273 in response to PKG activation with sildenafil in hearts of mice subjected to LV pressure overload. Taken together, our findings support a model in which PKGα inhibits cardiac remodeling through phosphorylation of cMyBP-C in the myocardium, through a process mediated by LZ domain binding. We interpret these findings to reveal cMyBP-C as a novel, myocardial-specific PKGα kinase substrate in the heart.

cMyBP-C as a Myocardial PKGα-Interacting Protein

Our identification of cMyBP-C as a PKGα-binding molecule supports an important role of PKGα in regulating myocardial-specific substrates. Pharmacological activation of PKGα attenuates LV remodeling in vivo,2 and this requires an intact

Figure 3. Protein kinase G α (PKGα) phosphorylation of cardiac myosin–binding protein-C (cMyBP-C) on antiremodeling residues. A, Schematic of cMyBP-C domains, with expanded regulatory phosphorylation motif on the M-domain from mouse and human sequences. Predicted PKGα phosphorylation sites on serines Ser-273, Ser-282, and Ser-302 with group-based phosphorylation score shown. Scores above 3.0 indicate high likelihood of PKGα phosphorylation. B, COS cells were transfected with full-length cMyBP-C and either wild-type PKGα (PKGα WT), or leucine zipper mutant PKGα (PKGα LZM), followed by 100 μmol/L 8Br-cGMP administration for 10 minutes. cMyBP-C phosphorylation was assayed by Western blot for Ser-273, Ser-282, and Ser-302 phosphorylation sites using phospho-specific antibodies. C, Quantitative densitometry is shown. Kruskal–Wallis 1-way ANOVA on ranks revealed global P of 0.057, 0.086, and 0.027 for Ser-273, Ser-282, and Ser-302 experiments, respectively. P values for multiple comparison testing are shown: *P < 0.05 vs LZM vehicle treated; **P < 0.05 vs WT vehicle and LZM vehicle; and †P < 0.05 vs WT vehicle, LZM vehicle, and LZM 8Br-cGMP. n=3 experiments. ADU indicates arbitrary densitometric unit. Adapted from Barefield and Sadayappan22 with permission of the publisher. Copyright © 2009, Elsevier.
remodeling. Furthermore, cMyBP-C attenuates LV remodeling and diastolic dysfunction and subsequent pathological LV function. Conditional deletion of cMyBP-C in mice leads to systolic dysfunction, although the role of these interactions in the chronic remodeling process has not been established.

PKGIα LZ domain. However, specific PKGIα substrates in the myocardium, which may mediate these effects, remain poorly understood. Previous work has identified ubiquitously expressed PKGIα substrates, such as the regulator of G protein signaling 2, as regulating the cardiac remodeling response in vivo. The role, if any, of PKGIα in the myocardium has been less well appreciated, and its role in the CM in regulating remodeling remains controversial. More recently, CM-specific PKGI substrates have been identified, such as troponin I and titin. Importantly, our screen also confirmed precipitation of troponin I with the PKGIα LZ domain. PKGI phosphorylation of these proteins may acutely regulate CM function, although the role of these interactions in the chronic remodeling process has not been established.

To our knowledge, our study identifies cMyBP-C as the first myocardial-specific antiremodeling molecule regulated by PKGIα. cMyBP-C functions in the sarcomere to regulate actin–myosin interaction and dynamics. Moreover, cMyBP-C is required for normal LV structure and function in vivo. Conditioned deletion of cMyBP-C in mice leads to systolic and diastolic dysfunction and subsequent pathological LV remodeling. Furthermore, cMyBP-C attenuates LV remodeling in response to pressure overload. In humans, structural mutations in cMyBP-C represent the most common genetic cause of hypertrophic cardiomyopathy and can also lead to dilated cardiomyopathy.

Our observations both in vitro and in cell culture that PKGIα directly phosphorylates the cMyBP-C M-domain on Ser-273, Ser-282, and Ser-302 support an important mechanism through which PKGIα may attenuate pathological remodeling. Accumulating evidence demonstrates that M-domain phosphorylation increases cMyBP-C interaction with the myosin head and improves CM systolic and diastolic function. Furthermore, genetic disruption of cMyBP-C M-domain phosphorylation leads to chronic remodeling, whereas genetic substitution of aspartate at these sites to mimic constitutive phosphorylation limits the chronic cardiac remodeling process in vivo. In human hypertrophied and failing hearts, cMyBP-C phosphorylation is decreased compared with hearts of normal donors. cMyBP-C M-domain phosphorylation increases rapidly after LV pressure overload but is reduced in the remodeled LV. Despite the apparent antiremodeling effect of cMyBP-C M-domain phosphorylation, kinases previously demonstrated to phosphorylate these sites, including protein kinase A, calmodulin kinase II, protein kinase C, and AKT, generally promote rather than inhibit pathological remodeling. Our observations therefore identify PKGIα phosphorylation on cMyBP-C M-domain sites as a novel upstream antiremodeling mechanism of cMyBP-C regulation.

Our finding that sildenafil increases cMyBP-C phosphorylation at Ser-273 in an in vivo model of cardiac remodeling, and HF further supports that PKGIα inhibits cardiac remodeling through regulation of cMyBP-C. Sildenafil inhibits cGMP catalysis by the cGMP-selective phosphodiesterase 5, thus increasing intracellular cGMP and activating PKGI. In the pressure overloaded LV, where phosphodiesterase 5 activity increases significantly, phosphodiesterase 5 inhibition
prevents excess cGMP breakdown and promotes PKGI kinase activity, leading to attenuated LV remodeling.2 Our study demonstrates that, in addition to previously reported antiremodeling effects, sildenafil treatment also increases cMyBP-C phosphorylation in pressure-overloaded hearts. This finding therefore provides evidence that pharmacological PKGI activation increases cMyBP-C phosphorylation on antiremodeling residues. Because cMyBP-C phosphorylation at Ser-273 inhibits cardiac remodeling,15,23,24 we interpret our findings to support that PKGI may normally inhibit pressure overload–induced remodeling through phosphorylation of cMyBP-C.

Significance of cMyBP-C as a Cardiac Myocyte–Specific Antiremodeling Substrate of PKGIα
Although PKGI inhibits cardiac remodeling in vivo, the downstream myocardial LZ-dependent substrates mediating this effect had not been systematically studied. Our findings that cMyBP-C functions as a CM-specific PKGIα substrate support an antiremodeling function of PKGIα within the CM. These findings also support the broader hypothesis that PKGIα separately regulates vasodilation and myocardial remodeling at least in part through independent sets of substrates. This is of substantial clinical relevance because trials of PKGIα-activating drugs, such as nesiritide, cinaciguat, and sildenafil, have been limited by vasodilating side effects arising from systemic PKGI activation.1 More recently, the compound LCZ696, which activates PKGI through inhibition of upstream natriuretic peptide degradation, has been shown to improve outcomes in HF, but also to induce excess hypotension.11 These clinical trial results further highlight the importance of understanding CM-specific PKGIα effectors, such as cMyBP-C, which might serve as therapeutic targets to selectively inhibit remodeling, but avoid excess hypotension arising from systemic PKGI activation.

Limitations
Our study has several limitations. First, although we demonstrate that PKGIα phosphorylates cMyBP-C on 3 critical antiremodeling sites, this study did not explore additional sites on cMyBP-C, which may also be regulated by PKGIα. Second, although we identified PKGI augmentation with sildenafil or cGMP as increasing cMyBP-C phosphorylation in vivo or in the cultured CM, we did not determine the degree to which inhibition of remodeling correlated with phosphorylation on a cell-by-cell or individual mouse level. Therefore, the degree to which the phosphorylation of cMyBP-C correlates with cellular remodeling remains untested. Third, it should be noted that in our initial screen, we determined the molecular species only in bands precipitating with the PKG1-59 LZ domain, but did not perform mass spectroscopy analysis on the corresponding region of negative control lanes that had no detectable bands. However, we circumvented this limitation.

Figure 5. In vivo regulation of cardiac myosin–binding protein-C (cMyBP-C) by protein kinase G Iα (PKGIα). A, Western blot for cMyBP-C Ser-273 phosphorylation and total cMyBP-C in hearts of mice subjected to 5 weeks of transaortic constriction and treated with either vehicle or 200 mg/kg per day sildenafil. B, Quantification of Ser-273 phosphorylation to total cMyBP-C. *P<0.05 by Mann–Whitney rank sum test; n=4 per group. C, Western blot for cMyBP-C Ser-273 and Ser-282 phosphorylation in adult rat ventricular myocytes treated for 1 h with 8-Br-cGMP (0, 10, and 100 μmol/L). Representative of 4 independent experiments. D, Quantification of Ser-273 and Ser-282 phosphorylation to total cMyBP-C from C. Kruskal–Wallis testing revealed global P<0.05 for each Ser-273 and Ser 282 phosphorylation sites. P values for multiple comparison by Dunn test are shown. Sil indicates sildenafil; and TAC, transaortic constriction. †P<0.05 vs 0 μmol/L control; n=3 to 4 per group.
by performing Western blot for cMyBP-C on identical pull-down experiments. These findings (Figure 2A) strongly support that cMyBP-C binds specifically with the WT PKGIα LCZ domain and not with the LZ mutant domain. Finally, although cMyBP-C phosphorylation decreases in the human failing heart,35 our study only examined the PKGIα regulation of cMyBP-C in animal and in vitro experiments. Therefore, although we speculate that PKGI-activating drugs, such as nesiritide, nitrates, sildenafil, and LCZ696, inhibit remodeling by inducing cMyBP-C phosphorylation, this remains untested in humans.

In summary, our study provides the first identification of a myocardial-specific PKGIα antiremodeling substrate. Our findings provide the rationale for broader investigation of PKGIα LCZ substrates in the heart as a strategy to identify novel antiremodeling therapies.

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Disclosures
None.

References
Over the past decade, preclinical studies have identified that protein kinase G I (PKG) inhibits the process of pathological cardiac remodeling. PKG-activating drugs, including phosphodiesterase 5 inhibitors (sildenafil and tadalafil), nitric oxide donors (nitrates), guanylate cyclase stimulators/activators (riociguat), natriuretic peptides (nesiritide), and most recently neprilysin inhibitors (LCZ-696), have garnered interest as potential antiremodeling therapies for patients with heart failure. However, because PKG also induces vascular relaxation, these PKG-activating agents have been limited significantly by hypotension. Understanding PKG molecular substrates specific to the myocardium might therefore identify new therapeutic strategies to inhibit pathological remodeling yet avoid excess vasodilation. For this reason, we performed a molecular screen for novel myocardial PKGα kinase substrates. Our screen revealed a cardiac myocyte-specific protein, cardiac myosin–binding protein-C (cMyBP-C), as binding to PKGαt. We found that PKGαt binds with cMyBP-C in heart tissue and also phosphorylates cMyBP-C on 3 specific amino acids which, when phosphorylated, are known to limit pathological remodeling. In mice subjected to left ventricular pressure overload, sildenafil administration, which inhibits adverse remodeling, augmented myocardial cMyBP-C phosphorylation. From the clinical perspective, these findings support that promoting cMyBP-C phosphorylation might represent a way to target the PKGα antiremodeling effect but avoid excess hypotension. These findings also support the emerging clinical paradigm that PKG-activating drugs inhibit remodeling through signaling mechanisms specific to the cardiac myocyte rather than solely through reduction of afterload. This suggests that understanding novel cardiac myocyte–specific PKGα substrates, including cMyBP-C and possibly others, may provide new therapeutic targets for heart failure.
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