

Genetically Encoded Biosensors Reveal PKA Hyperphosphorylation on the Myofilaments in Rabbit Heart Failure

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Running title: β AR Signal on the Myofilaments in Heart Failure



Circulation
Research

ONLINE FIRST

Subject Terms:

Heart Failure

Basic Science Research

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In July 2016, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 13.27 days.

ABSTRACT

Rationale: In heart failure, myofilament proteins display abnormal phosphorylation, which contributes to contractile dysfunction. The mechanisms underlying the dysregulation of protein phosphorylation on myofilaments is not clear.

Objective: This study aims to understand the mechanisms underlying altered phosphorylation of myofilament proteins in heart failure.

Methods and Results: We generate a novel genetically encoded protein kinase A (PKA) biosensor anchored onto the myofilaments in rabbit cardiac myocytes to examine PKA activity at the myofilaments in responses to adrenergic stimulation. We show that PKA activity is shifted from the sarcolemma to the myofilaments in hypertrophic failing rabbit myocytes. In particular, the increased PKA activity on the myofilaments is due to an enhanced β_2 adrenergic receptor (β_2 AR) signal selectively directed to the myofilaments together with a reduced phosphodiesterase activity associated with the myofibrils. Mechanistically, the enhanced PKA activity on the myofilaments is associated with downregulation of caveolin-3 in the hypertrophic failing rabbit myocytes. Reintroduction of caveolin-3 in the failing myocytes is able to normalize the distribution of β_2 AR signal by preventing PKA signal access to the myofilaments, and to restore contractile response to adrenergic stimulation.

Conclusions: In hypertrophic rabbit myocytes, selectively enhanced β_2 AR signaling toward the myofilaments contributes to elevated PKA activity and PKA phosphorylation of myofilament proteins. Reintroduction of caveolin-3 is able to confine β_2 AR signaling and restore myocyte contractility in response to β -adrenergic stimulation.

Keywords:

Heart failure, myofilament protein, PKA, adrenergic receptor, protein kinase A phosphorylation.

Nonstandard Abbreviations and Acronyms:

PDE	Phosphodiesterase
AR	adrenergic receptor
PKA	protein kinase A
TnI	troponin I
SR	sarcoplasmic reticulum
PLB	phospholamban
AKAR	A-kinase activity biosensor
FRET	fluorescence resonant energy transfer
Cav3	caveolin-3
ISO	isoproterenol

INTRODUCTION

In animal hearts, elevated sympathetic activity stimulates the β -adrenergic signal to promote inotropy and lusitropy via the concerted PKA phosphorylation of substrates at multiple subcellular locations. PKA is spatially and temporally regulated through a set of A-kinase anchoring protein complexes.¹ As a result, the precise PKA phosphorylation of myofilament proteins such as cardiac troponin I (TnI) and myosin binding protein C (MyBP-C) and Ca^{2+} -handling proteins such as phospholamban (PLB) and ryanodine receptor on the sarcoplasmic reticulum (SR) is necessary to coordinate positive inotropic and lusitropic cardiac effects.^{2,3}

Chronic adrenergic signaling also promotes structural and functional remodeling in the myocardium, which contributes to cardiac hypertrophy and eventually heart failure in a variety of clinical conditions. A desensitized cardiac β AR signaling pathway,⁴ a hallmark of the failing heart, is associated with a reduced number of β_1 AR but not β_2 AR binding sites at the plasma membrane (PM). As a consequence, diminished β AR signaling may lead to lower PKA-mediated protein phosphorylation after adrenergic stimulation.⁵ The disturbed β AR signaling and reduced PKA phosphorylation result in blunted contractile response following administration of β AR agonists.⁶ Interestingly, recent progress suggests that β AR can continuously signal both at the cell surface⁷ and after catecholamine-induced endocytosis.⁸ These β AR signals may play a role in adaptive cardiac hypertrophic remodeling and promote compensatory contractility in the heart. Until now, it remains poorly understood how remodeling of β AR signaling affects PKA activity in different subcellular locations for PKA-dependent phosphorylation of substrates in failing myocytes.

Recent efforts have been put forward to understand β -adrenergic signaling at the sarcolemma and the SR due to their essential roles in calcium handling and arrhythmia in cardiac diseases. By using genetically encoded biosensors, studies show that adrenergic-induced PKA activity at the SR can be selectively inhibited by chronic insulin⁹ and the pro-inflammatory factor, prostaglandin E,¹⁰ both of which are elevated in clinical conditions associated with heart failure. Moreover, in hypertrophic failing myocytes isolated from a transaortic constriction mouse model, the adrenergic stimulation-induced cAMP activity is reduced at the PM¹¹ and the SR,¹² consistent with depressed PKA phosphorylation of PLB.¹³ In contrast, much less is known about the regulation of PKA activity and PKA phosphorylation of substrates on the myofilaments during cardiac remodeling and disease development. A disturbed PKA phosphorylation of myofilament proteins such as TnI and MyBP-C alters their calcium binding sensitivity, which directly contributes to inotropic and lusitropic dysfunction in the heart.¹⁴

Here, we develop a novel genetically encoded PKA biosensor that is anchored on the myofibrils to probe PKA activity in cardiac myocytes from a rabbit hypertrophic heart failure model.¹⁵ Our data show that while the β_1 AR-induced PKA signal is detected at the myofilaments as well as the sarcolemma and the SR in healthy myocytes, the β_2 AR-induced PKA signal is restricted along the sarcolemma and the SR, and does not have access to the myofilaments. However, in hypertrophic failing rabbit myocytes, stimulation of β_2 AR promotes a strong PKA activity at the myofilaments. The emerging β_2 AR signal is associated with down regulation of caveolin-3 in hypertrophic failing myocytes, a key structural protein of caveolae that confines the β_2 AR signal.¹⁶ Thus, in failing myocytes stimulation of the β_2 AR promotes strong PKA activity and PKA phosphorylation of TnI. Moreover, we demonstrate that re-introduction of caveolin-3 normalizes the distribution of the β -adrenergic signal, and restores contractile response to adrenergic stimulation.

METHODS

Experimental animals, cardiac myocyte isolation and culture.

The animal care and experimental protocols followed US National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committees of the University of California at Davis. HF was induced in New Zealand White rabbits by combined aortic insufficiency and stenosis as previously described.¹⁵

Fluorescence resonance energy transfer (FRET) measurements.

Images were acquired using a Leica DMI3000B inverted fluorescence microscope (Leica Biosystems, Buffalo Grove, IL) with a 40X oil-emersion objective lens and a charge-coupled device camera controlled by Metafluor software (Molecular Devices, Sunnyvale, CA). Fluorescence images for FRET analysis were carried out as described previously.¹⁷

Statistical analysis.

Statistical analysis was performed using GraphPad Prism 6 software (La Jolla, CA).

Expanded methods can be found online.



RESULTS

Novel biosensors reveal highly localized β_2 AR-induced PKA activity that does not reach the myofilaments in healthy rabbit cardiac myocytes.

To analyze the dynamics of PKA activity on the myofilaments, we took a novel strategy to anchor FRET-based A-kinase activity reporter 3 (AKAR3)¹⁷ onto the myofilaments (MFs) by linking AKAR3 to the C-terminus of troponin T (MF-AKAR3, Figure 1A). MF-AKAR3 was used together with two other targeted PKA biosensors developed previously: PM-AKAR3 is linked to a PM targeting sequence from Kras and SR-AKAR3 is linked to the transmembrane domain of PLB.^{10,18} Confocal analysis of rabbit adult cardiac myocytes shows that the biosensors display proper subcellular distribution, co-localizing with specific markers for subcellular organelles (Figure 1B). The introduction of biosensors does not affect adrenergic stimulation-induced PKA phosphorylation of endogenous troponin I (TnI, Figure 1C) or PLB.^{10,18} These data indicate that the molecular pathways are preserved upon the expression of biosensors, and the targeted probes are suitable for analysis of PKA activity in the microdomains of adult cardiac myocytes.

We then further characterized the AKAR3 biosensors in response to a set of incremental doses of β -adrenergic agonist isoproterenol in adult rabbit myocytes. Stimulation of myocytes with isoproterenol induces dose-dependent increases in FRET ratio of the PM, SR and MF-anchored AKAR3 biosensors; and the maximal responses are higher at the PM than those at the SR and MF (Figure 1D and Supplemental Figure I). The responses of PM-AKAR3 are also more sensitive to isoproterenol stimulation than those of SR-AKAR3 and MF-AKAR3 (Figure 1D and 1E, and Supplemental Figure I). This data is consistent with previous studies showing that PKA phosphorylation of substrates at the PM is more sensitive to lower doses of isoproterenol stimulation than those at the intracellular compartments.¹⁹ The isoproterenol-induced maximal responses are not due to saturation of individual biosensors since co-treatment of myocytes with forskolin and IBMX induces higher increases in FRET ratios of biosensors than those induced by isoproterenol, respectively (Figure 1F). Notably, the isoproterenol-induced maximal responses in these localized biosensors are equivalent when normalized by those induced by co-treatment of forskolin and IBMX (Figure 1G), suggesting that these biosensors are functionally comparable in response to adrenergic stimulation in rabbit myocytes.

To analyze the alteration of local PKA dynamics at the myofilaments in failing heart, we used cardiac myocytes isolated from rabbits with hypertrophic heart failure (HF) induced by combined aortic insufficiency and stenosis.¹⁵ Myocytes from age-matched rabbits with sham surgery (SHAM) were used as controls. In age-matched SHAM myocytes, stimulation of the β AR with a saturated dose of isoproterenol (100 nmol/L) leads to an increase of PKA activity, which is completely blocked by β_1 AR selective antagonist CGP20712A, but not by β_2 AR selective antagonist ICI118551 (Figure 2A). Accordingly, stimulation of SHAM myocytes with isoproterenol significantly promotes PKA phosphorylation of troponin I (TnI) at serine 23/24, which is also blocked by CGP20712A, but not by ICI118551 (Figure 2B). However, stimulation with isoproterenol promotes a stronger PKA activity associated with the myofilaments in failing myocytes than SHAM cells (Figure 2C and 2D). Notably, stimulation of the β_2 AR also induces a strong increase in PKA activity in failing myocytes (Figure 2C and 2D). Consistent with the FRET measurement of PKA activity, stimulation of the β_2 AR promotes a strong increase in PKA phosphorylation of TnI in HF myocytes but not in SHAM controls (Figure 2E). These data indicate that in HF myocytes, the β_2 AR gains an ability to promote a strong PKA signal on the myofilaments.

β AR-induced PKA activity is diminished at the SR and the PM in failing rabbit myocytes.

We also examined the alteration of local PKA dynamics at the SR in failing myocytes, an important intracellular organelle that regulates calcium signaling for myocyte contraction. In SHAM myocytes, stimulation of the β AR promotes a significant increase in PKA activity at the SR, which is inhibited by β_1 AR antagonist CGP20712A, but not by β_2 AR antagonist ICI118551 (Figure 3A). Consistent with the FRET-based measurement of PKA activity, stimulation of SHAM myocytes with isoproterenol promotes PKA phosphorylation of PLB (PLB) at serine 16, which is completely blocked by CGP20712A, but not by ICI118551 (Figure 3B). However, stimulation with isoproterenol promotes a weaker PKA activity at the SR in failing myocytes than SHAM cells (Figure 3C and 3D). Further analysis reveals that the β_1 AR-induced PKA activity at the SR is reduced whereas the β_2 AR-induced PKA activity at the SR is slightly increased (Figure 3C and 3D). Consistently, the adrenergic-induced PKA phosphorylation of PLB is diminished in HF myocytes compared to those in SHAM controls. In both SHAM and HF cells, the β_1 AR acts as the primary receptor subtype in adrenergic stimulation (Figure 3E). These data indicate that in HF myocytes, the β AR-induced PKA activity is diminished at the SR for substrate phosphorylation.

In comparison, stimulation of β AR promotes a robust increase in PKA activity at the PM in SHAM myocytes, which is significantly decreased in HF myocytes (Figure 4A-4C). Inhibition of either β_1 AR with CGP20712A or β_2 AR with ICI118551 attenuates PKA activity induced by isoproterenol, with a stronger inhibition by the β_1 AR blocker CGP20712A (Figure 4B). Further analysis reveals that the reduced adrenergic response at the PM in HF myocytes is primarily due to a diminished β_1 AR-stimulated PKA activity (Figure 4A and 4D). These data suggest that the β AR-induced PKA activity is diminished at the PM in failing myocytes.

Redistribution of phosphodiesterase (PDE) activity contributes to elevated PKA activity on the myofilaments in failing heart.

Recent studies have also revealed essential roles of alteration in PDE expression in remodeling of adrenergic signal in HF myocytes.^{11, 20} We next studied the involvement of various cAMP PDEs on the modification of local PKA activity in HF myocytes. In SHAM myocytes, inhibition of PDE3 with cilostamide induces a strong increase in PKA activity at the myofilaments and the SR, but not at the PM (Figure 5A-5C). In comparison, inhibition of PDE4 with rolipram induces a strong response in PKA activity at the PM, and to a lesser extent, at the myofilaments and the SR (Figure 5D-5F). Consistent with these

observations, PDE3 displays co-localization with the myofilament binding protein actin whereas both PDE4D and PDE4B display co-localization with the plasma membrane marker caveolin-3 (Supplemental Figure II). In HF myocytes, the myofilament-associated PDE3 activity is decreased (Figure 5A and 5G) whereas the PM-associated PDE3 activity is increased relative to SHAM controls (Figure 5C and 5I), suggesting a shifted distribution of PDE3 activity from the myofilaments to the sarcolemma. In comparison, the PM-associated PDE4 activity in HF myocytes is decreased relative to SHAM controls (Figure 5F and 5I). Meanwhile, inhibition of PDE4 induces a small increase in PKA activity at the SR in SHAM myocytes, which is enhanced in HF cells (Figure 5E and 5H). However, the SR-associated PDE3 activity is not significantly altered in HF cells (Figure 5B and 5H). In addition, inhibition of PDE2 with erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) induces a small increase in PKA activity at the PM and myofilaments in SHAM myocytes; the ENHA-induced response is increased at the PM in HF cell whereas the ENHA-induced response is reduced at the myofilaments in HF cells (Supplemental Figure III). While the PDE2 activity at the SR is minimal in SHAM myocytes, it is increased in HF cells (Supplemental Figure III).

Together, in healthy rabbit myocytes, the PKA activity at the PM is under predominant control of PDE4 whereas PDE3 hydrolyzes preferentially a pool of cAMP in the vicinity of the myofilaments (Figure 5). In HF rabbit myocytes, the presence of PDE4 activity at the PM is reduced while the activity of PDE3 and PDE2 become more prominent. In contrast, the activity of PDE3 and PDE2 is decreased at the myofilaments (Figure 5 and Supplemental Figure III). These data indicate dysregulation of these hydrolytic enzymes in HF myocytes. Accordingly, western blotting analysis shows a reduction of PDE3 and an upregulation of PDE4 expression in left ventricles following HF (Figure 6A). Moreover, PDE4 is shifted to internal compartments whereas PDE3 is enriched at the sarcolemma region in HF myocytes relative to SHAM controls (Supplemental Figure IV). The membrane-enriched PDE3 does not co-localize with β_2 AR in HF myocytes (Supplemental Figure IV). Therefore, in HF myocytes, PDEs may not be effectively coupled to adrenergic receptors. Indeed, while inhibition of PDE4 and PDE3 potentiates adrenergic stimulation-induced PKA phosphorylation of TnI in SHAM myocytes, the effects of these PDE inhibitors are diminished in the HF cells (Supplemental Figure V).

In a separate set of experiments, we applied PKA inhibitors in the FRET measurement to assess the baseline subcellular PKA activity in HF and SHAM myocytes (Supplemental Figure VI). In SHAM myocytes, we detect a significant reduction of PKA activity by H89 at the myofilaments and the SR, but minimal change in PKA activity at the PM (Supplemental Figure VI). In HF myocytes, the H89-sensitive PKA activity at the PM and the SR does not change (Supplemental Figure VI). However, the H89-sensitive PKA activity at the myofilaments is increased in HF cells, indicating an elevation of baseline PKA activity associated with myofilaments in HF myocytes (Supplemental Figure VI).

Reintroduction of caveolin-3 restores distribution of β AR-induced PKA signal, PKA phosphorylation, and contractility in failing myocytes.

β_2 AR is enriched in the caveolae of cardiac myocytes, which confines the receptor action to the local PM domains. Disruption of caveolae leads to enhanced β_2 AR stimulation of ion channels at the PM.¹⁶ Previous studies have demonstrated that the expression of caveolin-3 is decreased in different mouse and human cardiac diseases including heart failure and hypertrophy.²²⁻²⁴ Loss of caveolin-3 is associated with redistribution of β_2 AR signal on the PM in failing myocytes.²⁵ Here, we reveal an 80% reduction of protein expression of caveolin-3 in the failing rabbit myocytes. This reduction is associated with an increase of PKA phosphorylation of TnI (Figure 6A). Caveolin-3 has a scaffolding domain (C3SD) that is critical for the structure and function of caveolae. A membrane permeable peptide corresponding to C3SD modulates β_2 AR regulation of L-type calcium channel.²¹ We applied the membrane permeable C3SD peptide to healthy rabbit myocytes to examine whether inhibition of caveolin-3 promotes β_2 AR signal to the myofilaments. Treatment with C3SD significantly enhances β_2 AR-induced PKA activity at the

myofilaments but not at the SR (Figure 6B and 6C). In contrast, a scrambled peptide does not enhance β_2 AR-induced PKA activity at the MF. Consistent with the FRET-based measurement of PKA activity, C3SD significantly promotes β_2 AR-induced PKA phosphorylation of TnI at the myofilaments (Figure 6D). These data suggest that caveolin-3 plays an essential role in preventing the β_2 AR from signaling to the myofilaments; therefore, loss of caveolin-3 in HF myocytes may permit β_2 AR to signal to the myofilaments to increase PKA activity and substrate phosphorylation.

We sought to restore the expression of caveolin-3 in hypertrophic failing myocytes and to evaluate β AR signaling and PKA activity on the myofilaments. Reintroduction of caveolin-3 partially restores the distribution of PDEs (Supplemental Figure IV) and their activity in HF myocytes, including an increase in PDE4 activity at the PM and increases in PDE3 and PDE2 activity at the myofilaments (Supplemental Figure III and VII). Moreover, reintroduction of caveolin-3 partially normalizes the distribution of β_2 AR (Supplemental Figure VIII) and PKA activity at the subcellular compartments, including increases in PKA activity at the PM, and decreases in PKA activity at the SR and myofilaments after stimulation with isoproterenol (Figure 7A-7C). While the β_1 AR-induced PKA activity is increased at the PM, the β_2 AR-induced PKA activity is decreased throughout the myocytes. Notably, after reintroduction of caveolin-3, the β_2 AR-induced PKA activity does not reach the myofilaments; instead it is confined at the PM and the SR (Figure 7A-7C). Accordingly, the phosphorylation of TnI under β_2 AR stimulation in failing myocytes is significantly diminished after overexpression of caveolin-3 (Figure 7D). Finally, overexpression of caveolin-3 restores β -adrenergic-induced contractile shortening response in hypertrophic failing rabbit myocytes (Figure 7E).

DISCUSSION

The cardiac PKA activity is fine-tuned to ensure coordinated phosphorylation of substrates in different compartments for excitation-contraction (E-C) coupling at baseline and after adrenergic stimulation. In this study, we have revealed a structural-functional remodeling in hypertrophic failing rabbit myocytes that links the redistribution of β AR signal to enhanced PKA phosphorylation of myofilament proteins (Figure 8). While the β_2 AR-induced signal is restricted along the sarcolemma and the SR in healthy myocytes, an augmented β_2 AR signal associated with loss of caveolin-3 is selectively directed to the myofilaments in hypertrophic failing cells. This augmented β_2 AR signal, together with reduced PDE activity associated with myofibrils, promotes strong PKA activity and PKA phosphorylation of the myofilament protein TnI in hypertrophic failing myocytes. Moreover, reintroduction of caveolin-3 is able to normalize the distribution of adrenergic signaling and restores contractile response to adrenergic stimulation in hypertrophic failing rabbit myocytes.

A tonic PKA activity is maintained by adenylyl cyclase-dependent cAMP production and phosphodiesterases-dependent cAMP hydrolysis.^{19,26} Our results show that PDE4 is the major enzyme that controls local PKA activity along the sarcolemma whereas PDE3 mediates primarily cAMP hydrolysis on the myofilaments (Figure 5). In agreement, both PDE4D and PDE4B display overlap with the membrane marker caveolin-3 (Supplemental Figure II), consistent with a recent report showing that both enzymes have a binding motif to caveolin-3;²⁷ loss of caveolin-3 may lead to loss of PDE4 activity at the sarcolemma. In contrast, PDE3 displays a co-localization with myofibrils (Supplemental Figure II), supporting its primary role in controlling local cAMP and PKA activity there (Figure 5). Meanwhile, we observe a high tonic PKA activity on the myofilaments, which is consistent with a relatively high PKA phosphorylation of MyBP-C and TnI in healthy cardiac tissues.²⁸

While the PKA activity is usually depressed in the late stage of human heart failure,^{29, 30} there are reports showing preserved or elevated PKA phosphorylation on TnI and PLB.^{31, 32} It is not completely understood how the cellular PKA activity in myocytes evolves during the course of heart failure development. Here, we observe a redistribution of PKA activity in hypertrophic failing myocytes, including an increased PKA activity at the myofilaments. In agreement, a recent report also shows that PKA phosphorylation of myofilament proteins is elevated in the spontaneous hypertensive rat model.³³ It is therefore possible that PKA activity is redistributed as part of the remodeling of β AR signaling during the compensatory stage before a general depression of PKA activity at the late stage of heart failure. In a classic view, β ARs are desensitized in HF due in part to the reduced β_1 AR density at the cell surface,³⁴ rendering the heart unable to respond to catecholamine stimulation⁶. However, recent progress suggests that β ARs can continuously signal at the cell surface⁷ and after catecholamine-induced endocytosis.⁸ Studies show that β ARs are accumulated at the endosome under chronic elevation of sympathetic activity in HF.³⁵ These β ARs may signal from endosomes, offering a potential mechanism on redistribution of PKA activity from the PM to the myofilaments in failing myocytes (Figure 2 and 4). Further analysis reveals that the diminished β_1 AR signaling contributes to lower PKA activity at the sarcolemma (Figure 4); which is consistent with previous observations that β_1 AR density is downregulated in this rabbit HF model.³⁶ In comparison, β_2 AR density is not reduced;³⁶ rather an augmented β_2 AR signal may be preferentially directed to the myofilaments. Moreover, the shifted PKA activity can be further exacerbated by relocation of PDE3 and PDE2 activity away from the myofilaments to the sarcolemma (Figure 5 and Supplemental Figure III). Together, these observations suggest that the β AR signal undergoes relocation intracellularly during the development of heart failure. Further studies will likely yield novel information on β AR-PKA signal remodeling in subcellular compartments in models of cardiac disease, which may offer insight into cardiac hypertrophic remodeling as well as compensatory contractility in the heart.

Detubulation and loss of caveolae are two prominent features during cardiac structural remodeling; these alterations can in turn promote signal remodeling in cardiac myocytes. While the β_2 AR is confined in the tubular region in healthy myocytes, chemical disruption of caveolae with a cholesterol-depleting agent, methyl- β -cyclodextrin, results in > 60% increase in β_2 AR-dependent PKA phosphorylation of PLB and TnI, as well as lusitropic responses in adult myocytes.³⁷ In addition, a membrane permeable peptide representing the scaffolding domain of caveolin-3 (C3SD) leads to reduction of β_2 AR-dependent PKA phosphorylation of L-type calcium channel at the PM.²¹ Detubulation and loss of caveolae in hypertrophic failing myocytes isolated from a mouse model yield broad distribution of β_2 AR signaling along the plasma membrane crest.^{25, 37} The loss of close proximity of β_2 AR to caveolin-3-associated PDE4²⁷ can also permit a much broader reach of receptor signaling outside of the T-tubular region in failing cells.²⁵ In this study, we have observed an amplified β_2 AR signal to the myofilaments after treatment with C3SD to promote PKA phosphorylation of TnI. Together, these observations suggest that inhibition of caveolin-3 leads to a signal shift from the PM to intracellular compartments. Moreover, we observed an amplified β_2 AR signal associated with loss of caveolin-3 in failing rabbit myocytes, which preferentially propagates toward the myofilaments (Figure 2 and 6). These data, for the first time, link the redistribution of β_2 AR to amplified PKA signal at the myofilaments in failing myocytes. Furthermore, reintroduction of caveolin-3 in HF myocytes can increase caveolae density,³⁸ which may facilitate the development and extension of T-tubules³⁹ and enrichment of β_2 AR to the T-tubule (Supplemental Figure VIII). Reintroduction of caveolin-3 may also recruit PDE4 enzymes to the proximity of the β_2 AR local domain²⁷ and Supplemental Figure II and IV) to confine β_2 AR signals in local subcellular environments.^{25, 40} Consequently, reintroduction of caveolin-3 effectively prevents β_2 AR from sending a signal to the myofilaments in rabbit failing myocytes and from promoting PKA phosphorylation of TnI (Figure 8). Moreover, reintroduction of caveolin-3 could normalize the functional relationship between β AR and the L-type Ca^{2+} channel at both T-tubules and caveolae.^{16, 21} As a result, the caveolin-3-expressing myocytes become more responsive to adrenergic stimulation to enhance contractility (Figure 7). Together, these data support the notion that caveolin-3 is essential in regulation of compartmented cardiac β AR signaling.^{25, 40}

Together, this study reveals that structural remodeling leads to β AR signal remodeling in failing myocytes, which yields a decrease in PKA activity at the PM, and an increase in β_2 AR-PKA activity and phosphorylation of substrates on the myofilaments in hypertrophic rabbit hearts. Our study suggests caveolin-3 as an essential regulator of subcellular adrenergic signal at the myofilaments in myocytes.

ACKNOWLEDGEMENT

We thank Matt L. Stein, Linda Talken, C. Blake Nichols, Ian Palmer, Max Bergman, Lisa J. Gilardoni, Maura Ferrero, and Yan Jiang for rabbit cell isolation.

SOURCES OF FUNDING

This study is supported by NIH grant R01 HL082846 to YKX, P01 HL80101 to DB, RO1 HL091071 to HHP, Veteran Affairs Merit Award from the Department of Veterans Affairs BX001963 to HHP and BX002900 to YKX, and Italian grant FIRB 2010 RBFR10URHP to FB. TW is a recipient of AHA predoctoral fellow; QS is a recipient of AHA postdoctoral fellow; and YKX is an AHA established investigator of the American Heart Association and a Shanghai Eastern Scholar.

DISCLOSURES

None.

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FIGURE LEGENDS

Figure 1. Generation of cardiac myofilament-targeted FRET biosensors. A) Schematic representation of subcellular-localized PKA AKAR3 biosensors. Yellow (YFP) and cyan (CFP) fluorescent proteins flank a PKA substrate and a forkhead-associated (FHA) domain that recognizes the phosphorylated PKA substrate. AKAR3 is linked to a Kras-derived sequence for plasma membrane (PM-AKAR3) localization, to a phospholamban (PLB)-derived sequence for sarcoplasmic reticulum (SR-AKAR3) localization, and to troponin T for myofilament (MF-AKAR3) localization. B) Representative confocal images of biosensor (green) expressed in young rabbit cardiac myocytes. Cells are immunostained with subcellular specific markers (red) for the SR (Ryanodine Receptor, RyR), the PM (Wheat Germ Agglutinin, WGA), and the MF (Phalloidin). Merge images confirm co-localization of fluorescent signals. C) Immunoblot analyses of PKA phosphorylation of troponin I (TnI) at serine 23/24 in response to isoproterenol (ISO) stimulation (1 or 100 nmol/L) in young rabbit myocytes infected with MF-AKAR3 probes. D) Young rabbit myocytes expressing PM-AKAR3, SR-AKAR3, and MF-AKAR3 are stimulated with a set of incremental doses of ISO. Time courses show AKAR3 FRET responses after stimulation with ISO. E) Normalized isoproterenol-induced dose response curves of AKAR3 biosensors (EC₅₀ PM-AKAR3 at 2.16×10^{-10} mol/L, SR-AKAR3 at 2.57×10^{-9} mol/L, and MF-AKAR3 at 3.89×10^{-9} mol/L). F) Maximal increases in AKAR3 FRET ratio after stimulation with ISO (1 μ M) or after co-treatment with forskolin (10 μ M) and IBMX (100 μ M). G) Normalized maximal FRET responses of individual AKAR3 biosensors against the increases induced by co-treatment with forskolin and IBMX, respectively.

Figure 2. A highly localized β_2 AR signal is not accessible to the myofilaments in SHAM rabbit cardiac myocytes. A) SHAM rabbit cardiac myocytes expressing MF-AKAR3 are stimulated with 100 nmol/L ISO (total), or in the presence of 100 nmol/L β_2 AR blocker ICI118551 (β_1 AR) or 300 nmol/L of the β_1 AR blocker CGP20712A (β_2 AR). The maximal increases in MF-AKAR3 are plotted in bar graph. The dash line indicates the maximal increases induced by forskolin (10 μ M) and IBMX (100 μ M). *** $p < 0.01$ by one-way ANOVA followed by post hoc Bonferroni test. B) Stimulation of SHAM cardiac myocytes with ISO in the presence of CGP20712A or ICI118551 for 5 min. The PKA phosphorylation of TnI at Ser 23/24 is detected in western blot, and the increases in phosphorylation of TnI are plotted in bar graph. N = 6, ** $p < 0.01$ by one-way ANOVA followed by post hoc Bonferroni test. C) Time courses of FRET responses are from SHAM and HF cardiac myocytes expressing MF-AKAR3 after stimulation of β ARs. D) Bar graph represents the maximal increases in MF-AKAR3 FRET ratio in panel C. * $p < 0.05$ and *** $p < 0.001$ by student t-test. E) Stimulation of SHAM and HF cardiac myocytes with ISO in the presence of CGP20712A or ICI118551 for 5 min. The PKA phosphorylation of TnI at Ser 23/24 is detected in western blot and quantified in bar graph. N = 6, ** $p < 0.01$ by one-way ANOVA followed by post hoc Bonferroni test).

Figure 3. Diminished β AR-induced PKA activity at the SR in failing cardiac myocytes A) SHAM rabbit cardiac myocytes expressing SR-AKAR3 are stimulated with 100 nmol/L ISO (total), or in the presence of 100 nmol/L β_2 AR blocker ICI118551 (β_1 AR) or 300 nmol/L of the β_1 AR blocker CGP20712A (β_2 AR). The maximal increases in SR-AKAR3 are plotted in bar graph. The dash line indicates the maximal increases induced by forskolin (10 μ M) and IBMX (100 μ M). ** $p < 0.01$ when compared to total by one-way ANOVA followed by post hoc Bonferroni test. B) Stimulation of SHAM cardiac myocytes with ISO in the presence of CGP20712A or ICI118551 for 5 min. The PKA phosphorylation of PLB at serine 16 is detected in western blot, and plotted in bar graph. N = 6, ** $p < 0.01$ by one-way ANOVA followed by post hoc Bonferroni test. C) Time courses of FRET responses are from SHAM and HF cardiac myocytes expressing SR-AKAR3 after stimulation of β ARs. D) Bar graph represents the maximal increases in SR-AKAR3 FRET ratio in panel C. * $p < 0.05$ by student t-test. E) Stimulation of SHAM and HF cardiac myocytes with ISO in the presence of CGP20712A or ICI118551 for 5 min. The PKA phosphorylation of PLB is detected in western blot and quantified in bar graph. N = 6, ** $p < 0.01$ and *** $p < 0.001$ by one-way ANOVA followed by post hoc

Bonferroni test.

Figure 4. Redistribution of β AR subtype activity at the PM in failing cardiac myocytes A) SHAM and HF rabbit cardiac myocytes expressing PM-AKAR3 are stimulated with 100 nmol/L ISO (total), in the presence of 100 nmol/L β 2AR blocker ICI118551 (β 1AR), or 300 nmol/L of the β 1AR blocker CGP20712A (β 2AR). Time courses show FRET responses from SHAM and HF cardiac myocytes expressing PM-AKAR3 after stimulation of β ARs. B-D) The maximal increases in PM-AKAR3 in SHAM and HF myocytes are plotted in bar graph. In panel B, the dash line indicates the maximal increases induced by forskolin (10 μ mol/L) and IBMX (100 μ mol/L). # $p < 0.05$ and ## $p < 0.01$ by one-way ANOVA followed by post hoc Bonferroni test. * $p < 0.05$ by student t-test.

Figure 5. Redistribution of PDE activity in failing cardiac myocytes. A-F) SHAM and HF rabbit cardiac myocytes expressing MF-AKAR3, SR-AKAR3, or PM-AKAR3 are treated with 10 μ mol/L rolipram (PDE4), or 1 μ mol/L cilostamide (PDE3). Time courses of FRET traces from SHAM and HF cardiac myocytes are shown. G-I) The maximal increases in AKAR3 FRET ratio in SHAM and HF myocytes are plotted in bar graph. * $p < 0.05$ and *** $p < 0.001$ by student t-test.

Figure 6. Inhibition of caveolin-3 promotes adrenergic signaling on the myofilaments in HF cardiac myocytes. A) Immunoblots show expression of caveolin-3, PDE3A, PDE4, TnI, PLB, and phospholemman (PLM), as well as pTnI at serine 23/24, pPLB at serine 16, and pPLM at serine 68 in SHAM and HF heart lysates. Bar graphs represent mean $\pm$ SEM. $N = 3$; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.01$ by student's t-test. B and C) Healthy young rabbit myocytes expressing SR-AKAR3 or MFAKAR3 are stimulated with 100nmol/L ISO and 300 nmol/L CGP20712A after incubation with 1 μ mol/L C3SD or scrambled peptide for 1 hour. Time courses of changes in AKAR3 FRET ratio are shown, and the maximal increases in FRET ratio are plotted in bar graph. *** $p < 0.001$ by one-way ANOVA followed by post hoc Bonferroni test. D) Healthy young rabbit myocytes are stimulated with 100 nmol/L ISO in the presence of 1 μ mol/L C3SD or scrambled peptide. PKA phosphorylation of troponin I at serine 23/24 is detected in western blots and quantified in bar graph. $N = 3$; ** $p < 0.01$ by one-way ANOVA followed by post hoc Bonferroni test.

Figure 7. Expression of caveolin-3 normalizes adrenergic signaling on the myofilaments in HF cardiac myocytes. A-C) SHAM or HF myocytes expressing MF-AKAR3, PM-AKAR3 or SR-AKAR3 together with caveolin-3 as indicated. Myocytes are stimulated with 100 nmol/L ISO, or in the presence of 100 nmol/L ICI118551 or 300 nmol/L CGP20712A. The maximal increases in FRET ratio are plotted in bar graph. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ by one-way ANOVA followed by post hoc Bonferroni test. D) HF myocytes expressing caveolin-3 are stimulated with 100 nmol/L ISO in the presence of 300 nmol/L CGP20712A. PKA phosphorylation of troponin I at serine 23/24 are detected in western blots, and quantified in bar graph. $N = 5$; * $p < 0.01$ by one-way ANOVA followed by post hoc Bonferroni test. E) SHAM and HF rabbit myocytes with or without expression of caveolin-3 are stimulated with ISO (100 nmol/L). Myocyte contractile shortening is plotted as bar graphs. * $p < 0.05$ by one-way ANOVA followed by post hoc Bonferroni test.

Figure 8. Schematic models on changes of local PKA signal in failing cardiac myocytes. In SHAM cardiac myocytes, β 1AR has a higher and further reaching response in all three subcellular compartments tested than β 2AR. β 2AR generates a highly localized response within the PM and the SR, but does not induce any significant PKA activity on the myofilaments. PKA activity at the PM microdomain is under predominant control of PDE4 whereas PDE3 preferentially hydrolyzes a pool of cAMP in the vicinity of myofilaments. In HF myocytes with reduced caveolin-3, the β AR-induced PKA activity at the PM and, to a lesser extent, at the SR is reduced. However, the β AR-induced PKA activity at the myofilament is increased due in part to augmented β 2AR signal and miss-localization of PDE3 away from the myofilaments. Reintroduction of caveolin-3 normalizes the distribution of β AR-induced PKA activity at the subcellular compartments, including increases in PKA activity at the PM, and decreases in PKA activity

at the myofilaments. In particular, the β 2AR-induced PKA activity is confined at the PM and the SR, and does not access the myofilaments.



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Novelty and Significance

What Is Known?

- Abnormal PKA phosphorylation of substrates on myofilaments directly contributes to inotropic and lusitropic dysfunction in heart failure.
- Adrenergic signaling is altered in heart failure myocytes, including down regulation of β_1 adrenergic receptor (AR)-induced signals and an increase in the β_2 AR-induced signal.

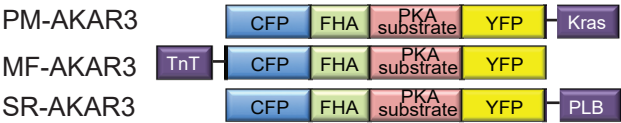
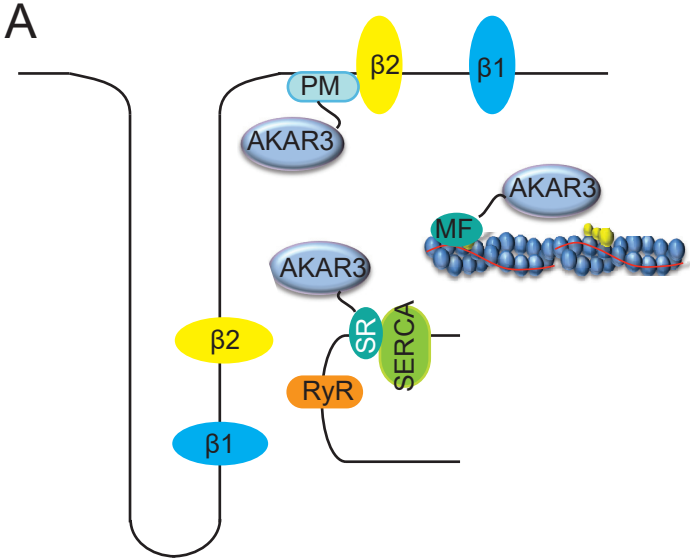
What New Information Does This Article Contribute?

- In heart failure myocytes, β_1 AR-induced signaling is selectively down regulated at the sarcolemma whereas β_2 AR-induced signaling gains access to myofilament.
- Reintroduction of caveolin 3 in heart failure myocytes prevents β_2 ARs from sending signal to myofilaments for substrate phosphorylation.
- Reintroduction of caveolin 3 normalizes β AR-induced contractile responses in heart failure myocytes.

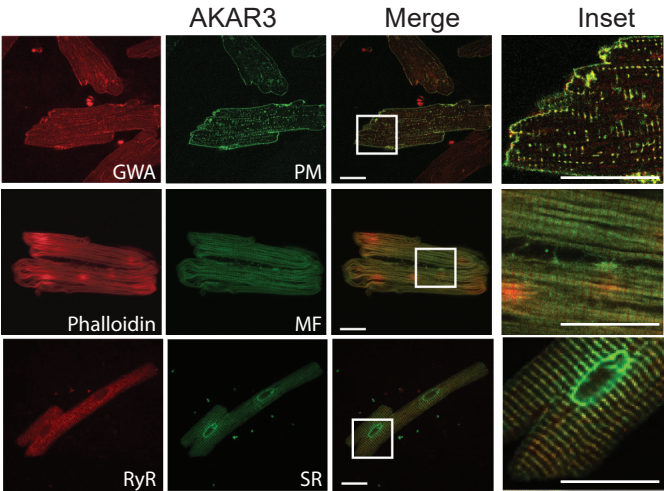
These studies reveal that β AR signaling undergoes remodeling in heart failure myocytes, which shifts the PKA activity from the cell surface to the intracellular compartments including the SR and myofilaments. In particular, β_2 AR-induced signals gain access to the myofilament, which contributes to abnormal PKA phosphorylation of TnI and contractile dysfunction. Preclinical studies to assess the effects of inhibition of β_2 AR signal on heart failure are indicated.

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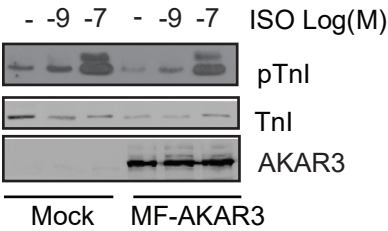
Figure 1



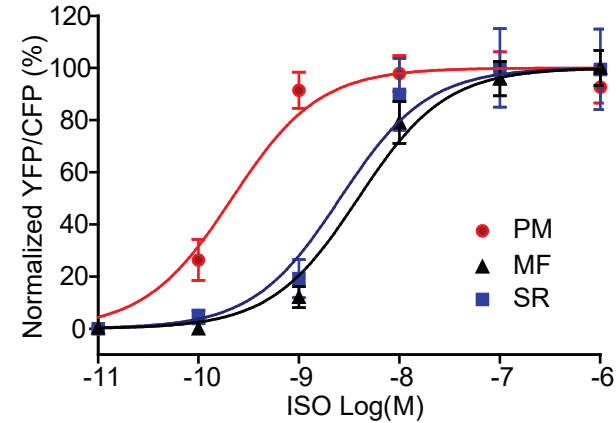
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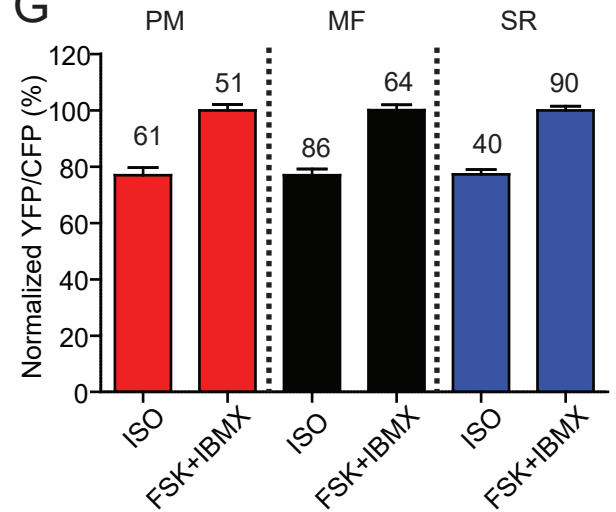
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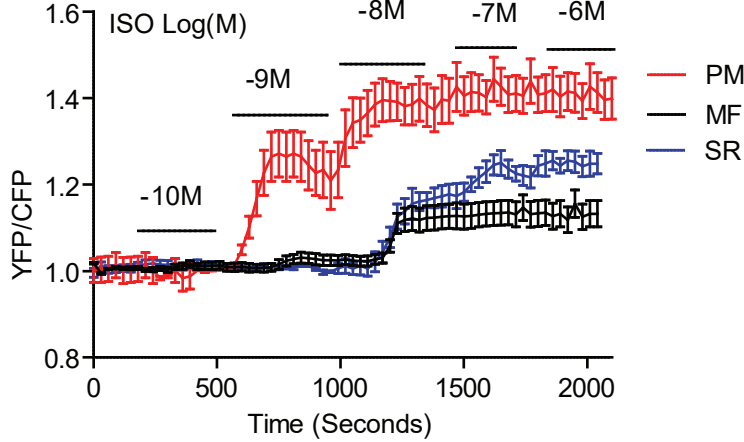
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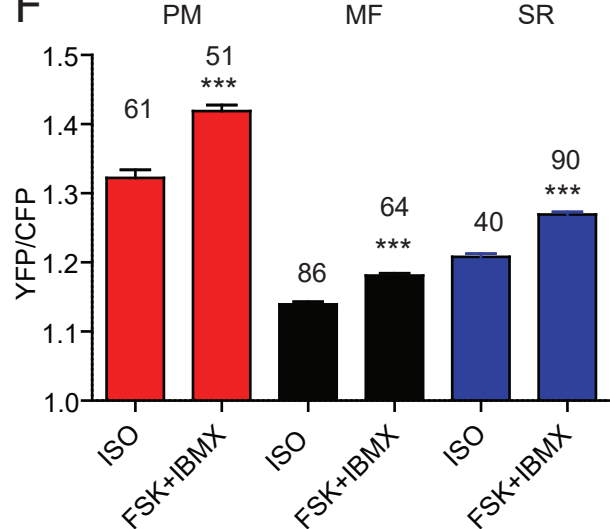


Figure 3

SR

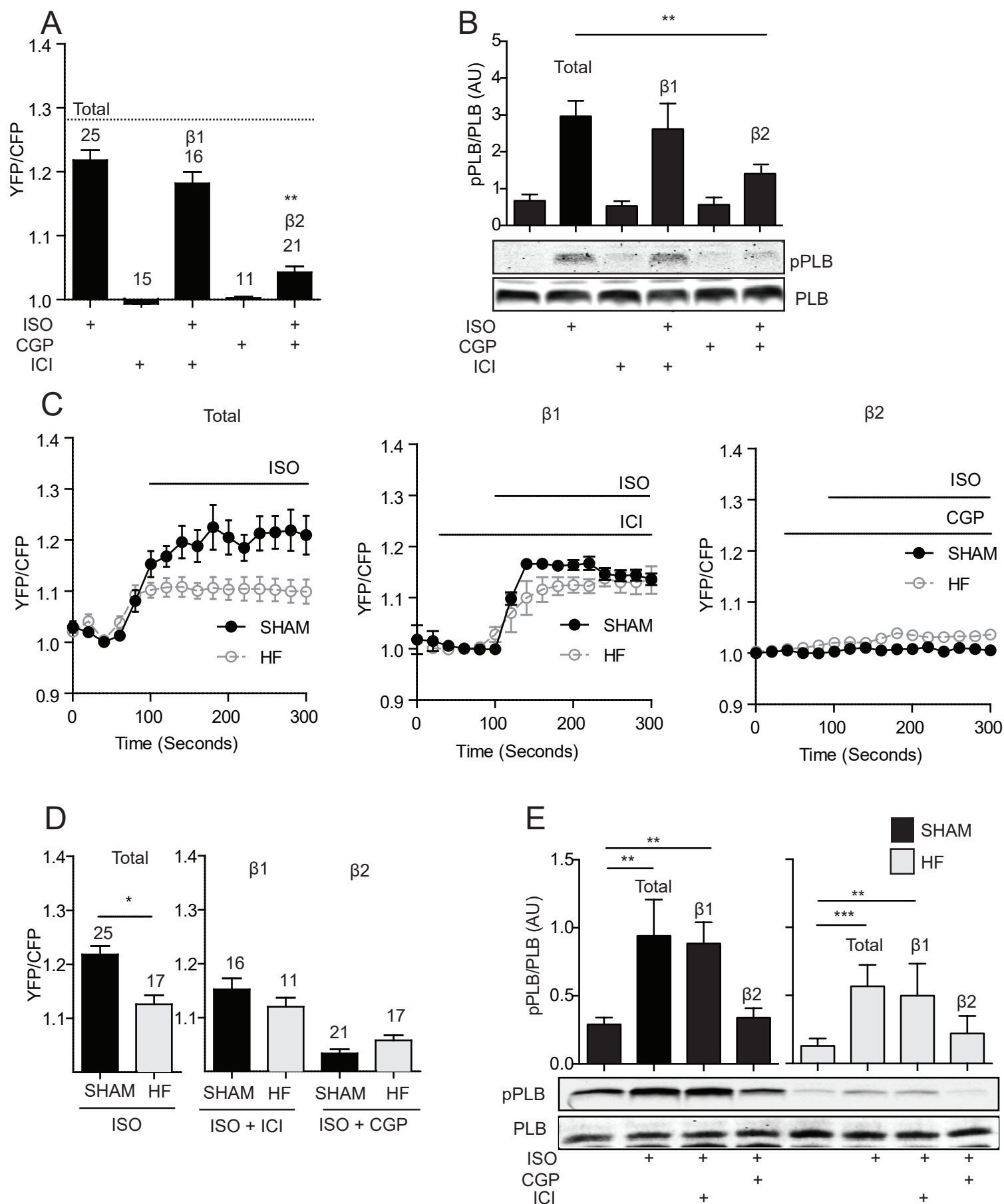


Figure 4

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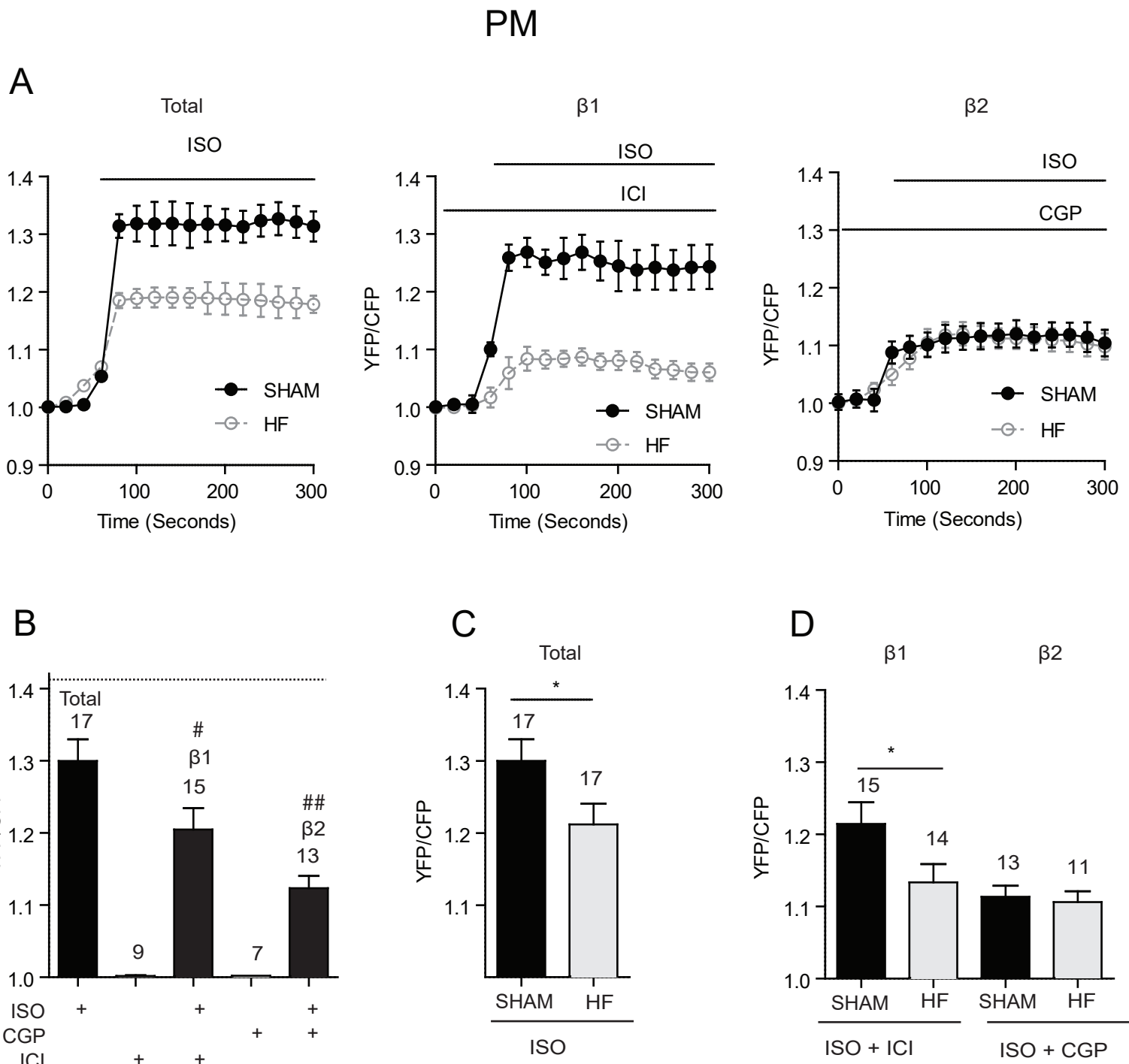


Figure 5

MF

SR

PM

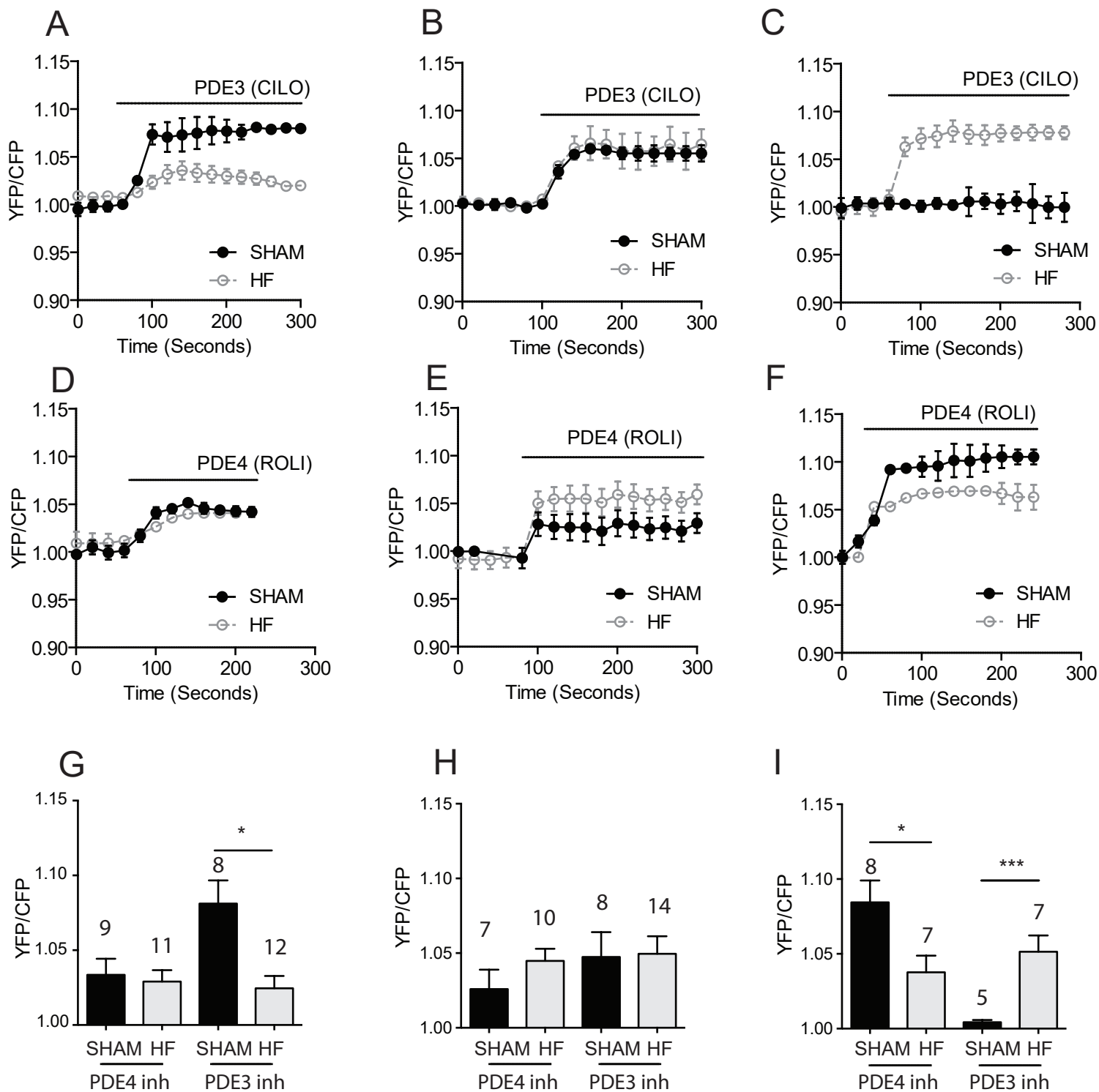


Figure 6

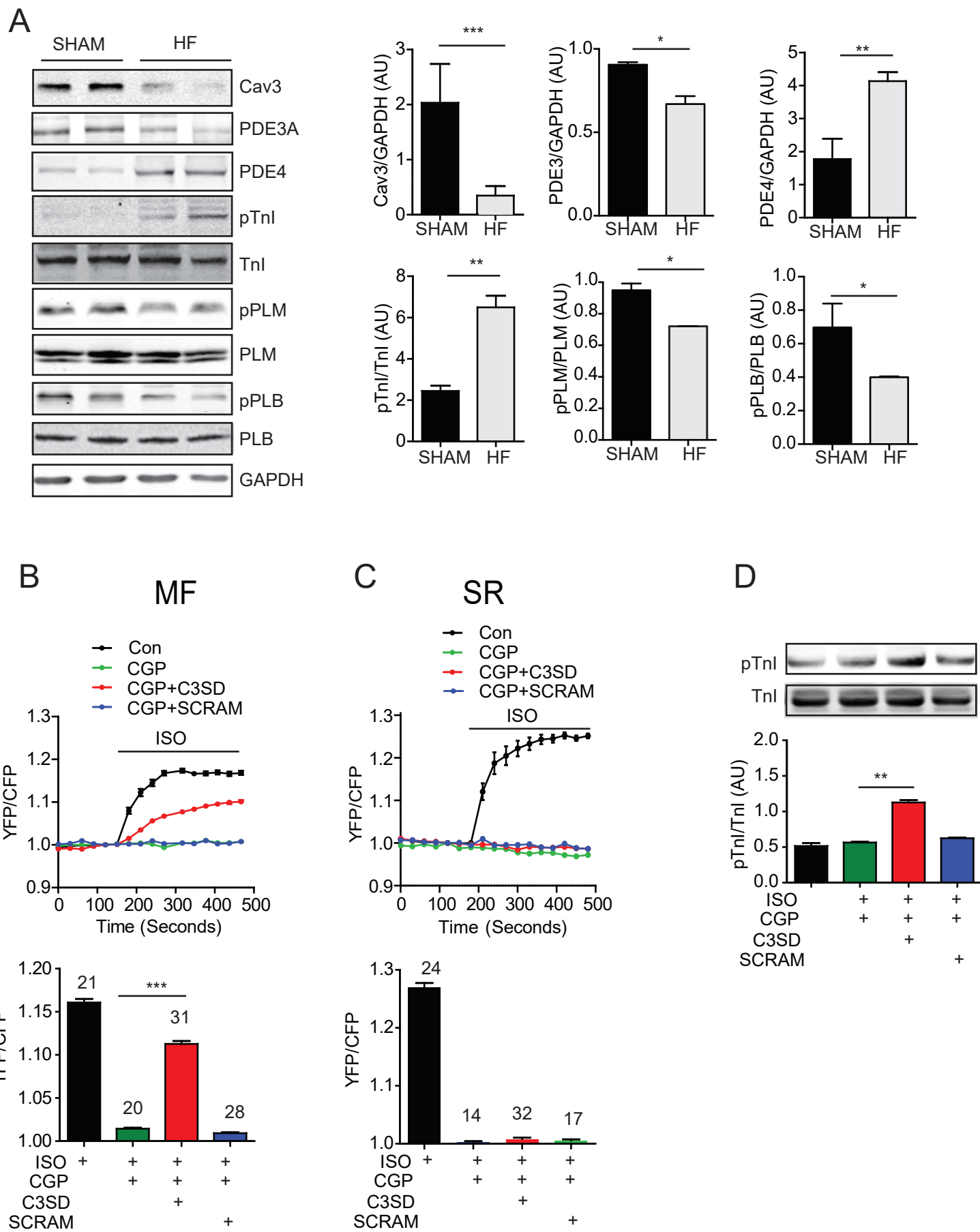
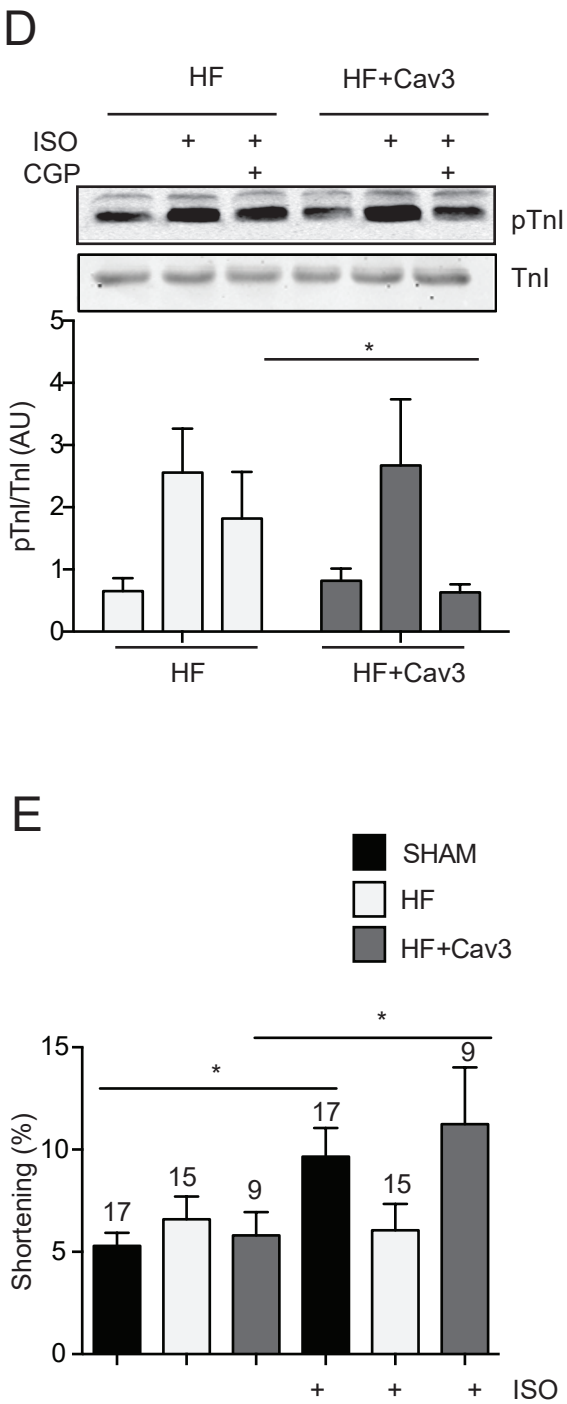
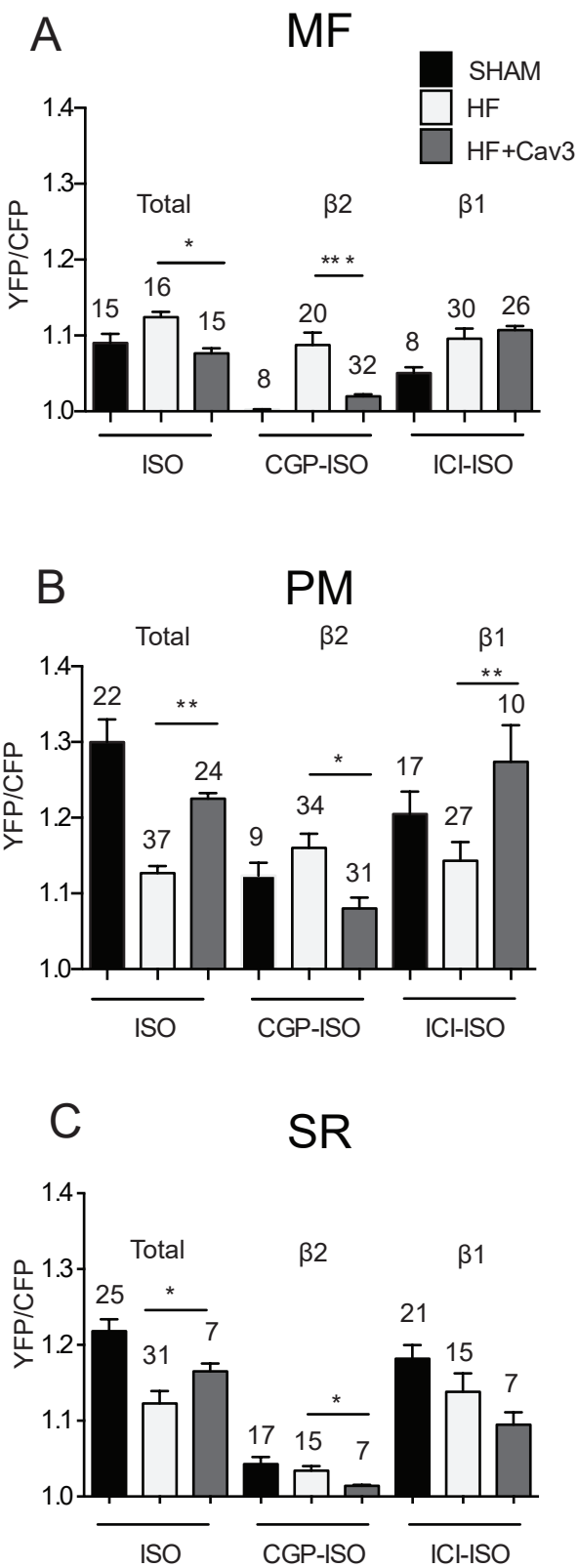
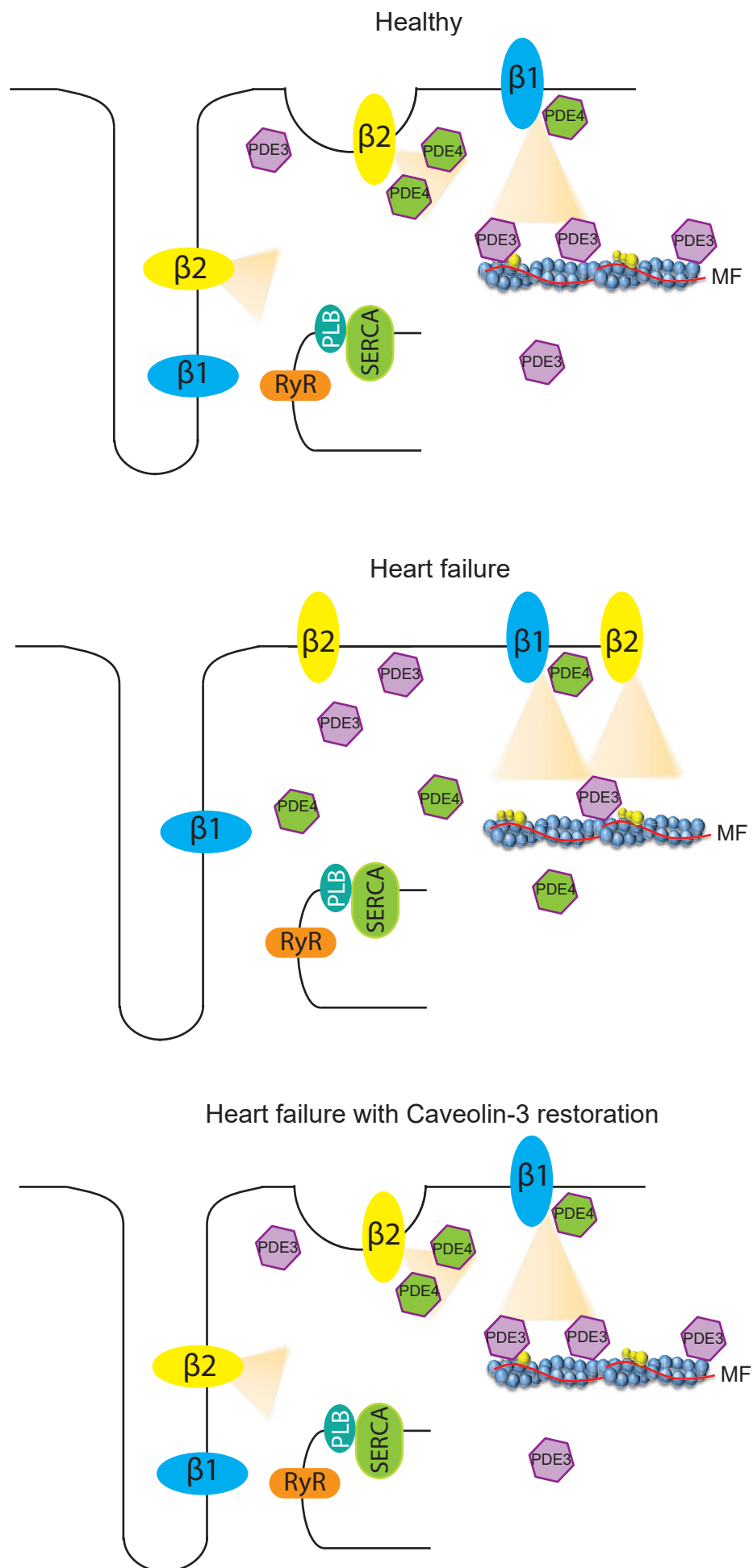


Figure 7



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Genetically Encoded Biosensors Reveal PKA Hyperphosphorylation on the Myofilaments in Rabbit Heart Failure

Federica Barbagallo, Bing Xu, Gopireddy Reddy, Toni West, Qingtong Wang, Qin Fu, Minghui Li, Qian Shi, Kenneth Ginsburg, William Ferrier, Andrea Isidori, Fabio Naro, Hemal Patel, Julie Bossuyt, Donald Bers and Yang Xiang

Circ Res. published online August 30, 2016;

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Supplement Materials.

Online expanded Methods

Development of PKA FRET-based biosensor and adenovirus construction

The regular PKA activity biosensor AKAR3¹ was linked to the C-terminus of cardiac troponin T (provided by Dr. Jianpin Jin, Wayne State University, OH) to generate a myofilament-anchored AKAR3, termed MF-AKAR3. The recombinant construct in pcDNA3.1 (Invitrogen, CA, USA) was verified by DNA sequencing. MF-AKAR3 cDNA was subsequently subcloned into a shuttle vector pAdTrack-CMV to generate recombinant adenovirus according to the manufacturer's instructions (Quantum Biotechnologies, Republic of South Africa). The PM-AKAR3 and SR-AKAR3 have been reported previously.^{2,3}

Experimental animals, cardiac myocyte isolation and culture

The animal care and experimental protocols followed US National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committees of the University of California at Davis. HF was induced in New Zealand White rabbits by combined aortic insufficiency and stenosis as previously described.⁴ HF progression was monitored by echocardiography and myocytes were isolated 7.1±1.9 months after aortic constriction when left ventricle end-systolic dimension exceeded 1.4 cm. Briefly, rabbits were sacrificed under general anesthesia (induction with propofol 2 mg/kg followed by 2-5% isoflurane in 100% oxygen). After thoracotomy, the heart was quickly excised and rinsed in cold nominally Ca-free MEM. The right atrium was removed and the aorta opened to visualize the left coronary ostia which was then cannulated using a 4F catheter.⁵ Perfusion of the left ventricle and left atrium was established before removal of the right ventricle free wall and application of a purse-string suture to secure the catheter in place. The remainder of the isolation procedure was then essentially as previously described.⁶

For myocyte cultures, cells were plated on natural mouse laminin (Life Technologies, Grand Island, NY) coated dishes in culture medium PC-1 (Lonza Walkersville, MD, USA) supplemented with 1% Penicillin-Streptomycin, PH 7.4, filtered) and infected, when specified, with PM-AKAR3, SR-AKAR3, or MF-AKAR3 biosensor together with GFP-caveolin-3 or caveolin-3 for at least 28 hours.

Immunostaining

Confocal microscopy was performed using Zeiss LSM700 microscope (Zeiss, Pleasanton, CA) equipped with a Plan-Apochromat 63X oil-immersion objective. For co-localization experiments, cells were fixed for 15 min with PFA 4%, rinsed 3 times with PBS and permeabilized with blocking solution (0.5% NP40, 2% goat serum) for 15 min. Cells were then washed and stained 2 hours with anti-RyR antibody (obtained from the DSHB, the University of Iowa, IA), anti-caveolin-3 (BD, NJ, USA #610420), anti-PDE3 (a gift from Yan Chen, University of Rochester) anti-PDE4B (a gift from Marco Conti, University of California at San Francisco) antibodies, anti-PDE4D (Ab14613) (Abcam, Cambridge, UK) followed by 1 hour of the secondary anti-mouse or anti-rabbit Alexa Fluor® 488 antibodies (A-21063 and A-11055, respectively) (Thermo Fisher,

MA, USA), or followed by Phalloidin Alexa Fluor® 568 Conjugate (A-12380) or Wheat Germ Agglutinin, Alexa Fluor® 555 Conjugate (W32464) (Thermo Fisher, MA, USA).

Western blotting

Left ventricular extracts or isolated cardiac myocytes were prepared in lysis buffer (25mM Tris HCl PH 7.6, 150 mM NaCl, 1% IGEPAL, 1% Sodium deoxycholate, 0.1% SDS, 1mM EDTA) with protease and phosphatase inhibitors (Na3F 100mM, Na2VO4 1mM, glycerol 1mM, NaP2O7 2.5 mM, leupeptin 10 µg/ml, PMSF 1mM, aprotinin 10 µg/ml), and protein concentration was measured by BCA assay (Pierce, IL). Lysates were resolved by SDS-PAGE before being transferred onto a PVDF membrane (Merck Millipore Ltd. MA, USA) and incubated overnight with primary antibodies followed by IRDye 680CW or 800CW secondary antibodies and acquired with an Odyssey scanner (LI-COR Biosciences Lincoln, NE, USA). The primary antibodies used for Western blotting were as follows: anti-β₂AR (sc-570) (SCBT, TX, USA), anti-PDE3A (1098-1115, NIH), anti-PDE4D (Ab14613) (Abcam, Cambridge, UK) anti-phospho-PLB (Ser16, Badrilla), anti-PLB (MA3-922) (Affinity Bioreagent, CO, USA), anti-phospho-troponin I (Ser23/24, #4004) and anti-troponin I (#4002) (Cell Signaling, MA, USA). Signal intensity was quantitated by Image Studio software version 2.1 (LI-COR Biosciences Lincoln, NE, USA).

Fluorescence resonance energy transfer (FRET) measurements

Images were acquired using a Leica DMI3000B inverted fluorescence microscope (Leica Biosystems, Buffalo Grove, IL) with a 40X oil-emersion objective lens and a charge-coupled device camera controlled by Metafluor software (Molecular Devices, Sunnyvale, CA). Fluorescence images were recorded by exciting the donor fluorophore at 430-455 nm and measuring emission fluorescence with two filters (475DF40 for cyan and 535DF25 for yellow). Images were subjected to background subtraction, and were acquired every 20 seconds with exposure time of 200 ms. The donor/acceptor FRET ratio was calculated and normalized to the ratio value of baseline. The binding of cAMP to AKAR3 increases CFP/YFP FRET ratio.¹

Adult cardiomyocyte contractility

Fresh isolated adult cardiomyocytes were infected with GFP or GFP-caveolin-3 adenovirus for 18 hours. Cells were then placed in a dish with 3 ml beating buffer (NaCl 120 mM, KCl 5.4 mM, NaH₂PO₄ 1.2mM, MgSO₄ 1.2 mM, HEPES 20mM, Glucose 5.5 mM, CaCl₂ 1mM, PH 7.1, filtered). Cells were paced at 1 Hz with voltage of 30 V using a SD9 stimulator (Grass Technology, Warwick, RI). Contractile shortening of myocytes were recorded on an inverted microscope (Zeiss AX10) at 20X magnification using Metamorph software (Molecular Devices, Sunnyvale, CA) with the following settings: 40 frames per second for 5 seconds, at an interval of 1 minute for a duration of 10 minutes. Isoproterenol (100 nM) was added into the dish after two movies were acquired. The percentage of myocyte fractional shortening (FS) was calculated as (maximal cell length – minimal cell length)/ maximal cell length * 100%. Analysis was performed using Metamorph.

Statistical analysis

All data were expressed as mean ± SEM. Statistical analysis was performed using GraphPad

Prism 6 software (La Jolla, CA). The sample size for each group was shown in the figure legends. The cells were from at least three sets of independent experiments. Differences between two groups were evaluated by 2-tailed Student's *t*-test; comparisons of multiple groups were performed using one-way ANOVA followed by post hoc Bonferroni test. $p < 0.05$ was defined as statistically significant.

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Supplemental Figure legend

Supplemental Figure I. Dose-dependent increases in AKAR3 FRET biosensors after stimulation of β AR in young adult rabbit myocytes. Rabbit myocytes expressing PM-AKAR3, SR-AKAR3, and MF-AKAR3 are stimulated with increasing doses of ISO. The maximal increases in different doses of ISO are plotted as dose response curves for individual biosensors, respectively.

Supplemental Figure II. Distribution of PDEs in SHAM and HF rabbit cardiac myocytes Immunostaining shows distribution between PDE4B or PDE4D (red) and caveolin-3 (green), and between PDE3A (green) and the myofilaments (phalloidin, red) in healthy, HF, or HF myocytes with caveolin-3 expression.

Supplemental Figure III. Redistribution of PDE2 activity in HF rabbit cardiac myocytes

Quantification of the maximal changes in FRET ratio in SHAM and HF myocytes with or without expression of caveolin-3. Cells expressing PM-AKAR3, SR-AKAR3 or MF-AKAR are stimulated with the PDE2 selective inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA, 1 μ mol/L). * $p < 0.05$ and ** $p < 0.01$ by one-way ANOVA followed by post hoc Bonferroni test.

Supplemental Figure IV. Distribution of PDEs in SHAM and HF rabbit cardiac myocytes A and B) Immunostaining shows distribution between PDE4D (red) and caveolin-3 (green), and between PDE3A (green) and the myofilaments (phalloidin, red) in SHAM, HF, or HF myocytes with caveolin-3 expression. C) HF rabbit myocytes expressing flag- β_2 AR are fixed for immunostaining to show distribution of between β_2 AR and PDE3A or PDE4D.

Supplemental Figure V. PDE regulates PKA-mediated phosphorylation of myofilaments in SHAM and HF rabbit cardiac myocytes. Immunoblots show PKA phosphorylation of TnI in SHAM or HF cells after stimulation with ISO (100 nmol/L, 5 min) in the presence of PDE2 inhibitor

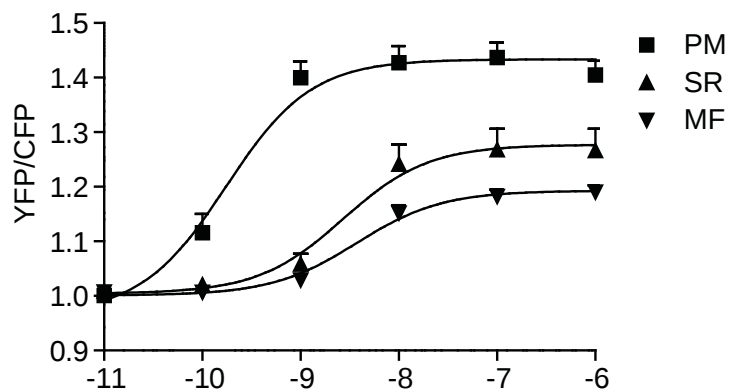
(EHNA 1 $\mu\text{mol/L}$), PDE3 inhibitor cilostamide (Cilo, 100 nmol/L), or PDE4 inhibitor Rolipram (Roli, 100 nmol/L). Bar graphs show mean $\pm$ SEM, N = 4, * $p < 0.05$ by one-way ANOVA followed by post hoc Bonferroni test.

Supplemental Figure VI. Detection of baseline PKA activity at the subcellular compartments in SHAM and HF rabbit myocytes. Data shows quantification of the maximal changes in FRET ratio in SHAM and HF rabbit myocytes. A) Young adult myocytes expressing MF-AKAR3 are stimulated with ISO (100 nmol/L) in the presence of PKA inhibitor myr-PKI (10 $\mu\text{mol/L}$), KT5720 (10 $\mu\text{mol/L}$) or H89 (10 $\mu\text{mol/L}$). *** $p < 0.001$ by one-way ANOVA followed by post hoc Bonferroni test. B) SHAM and HF rabbit myocytes expressing PM-AKAR3, SR-AKAR3 or MF-AKAR are treated with PKA inhibitor H89 (10 $\mu\text{mol/L}$). * $p < 0.05$ by student's *t*-test.

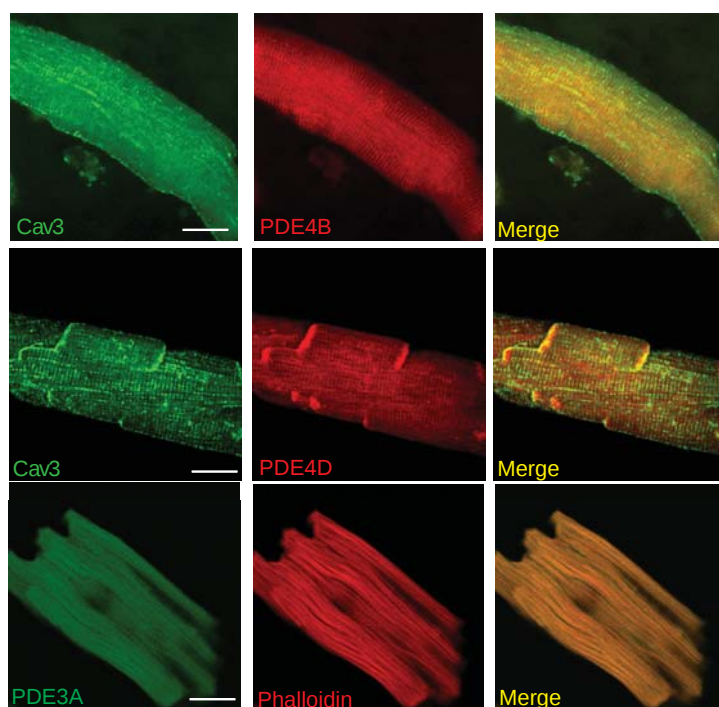
Supplemental Figure VII. Caveolin 3 modulates subcellular distribution of PDE3 and PDE4 activity in HF rabbit cardiac myocytes. Data shows quantification of the maximal changes in FRET ratio in SHAM myocytes and HF myocytes with or without expression of caveolin-3. Cells expressing PM-AKAR3 or MF-AKAR are treated with PDE3 selective inhibitor cilostamide (Cilo, 1 $\mu\text{mol/L}$) or PDE4 selective inhibitor Rolipram (Roli, 10 $\mu\text{mol/L}$). * $p < 0.05$ by student's *t*-test.

Supplemental Figure VIII. Localization of $\beta_2\text{AR}$ and caveolin-3 in HF rabbit cardiac myocytes. HF rabbit myocytes expressing flag- $\beta_2\text{AR}$ alone (lower panels) or together with caveolin-3 (upper panels) are fixed for immunostaining to show distribution of $\beta_2\text{AR}$ and caveolin-3.

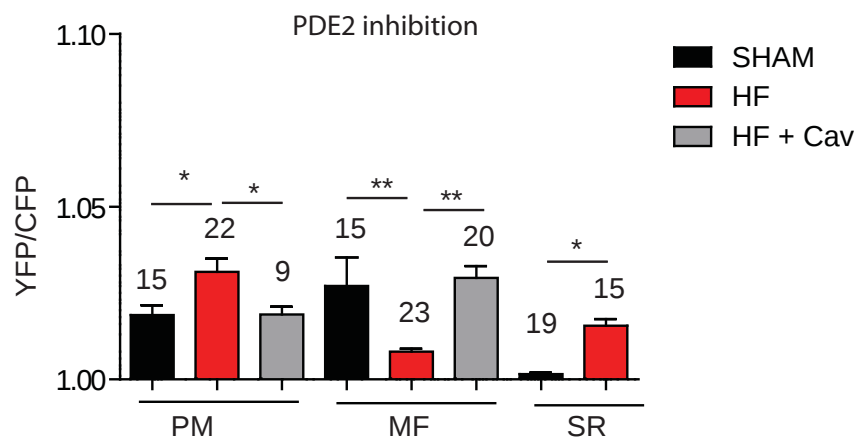
Supplemental Figure I



Supplemental Figure II

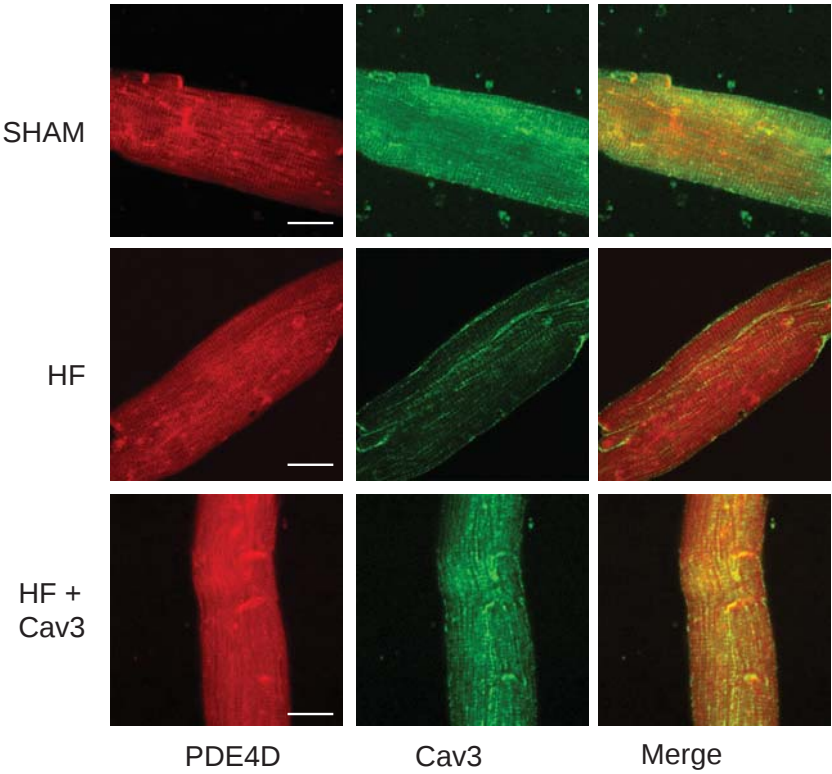


Supplemental Figure III



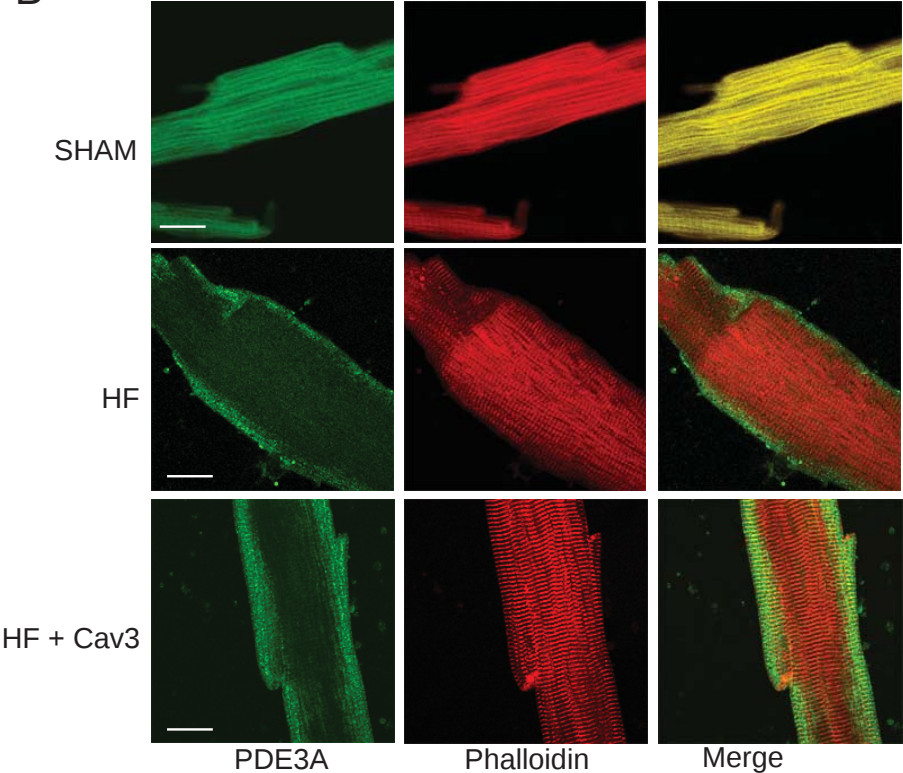
Supplemental Figure IV

A

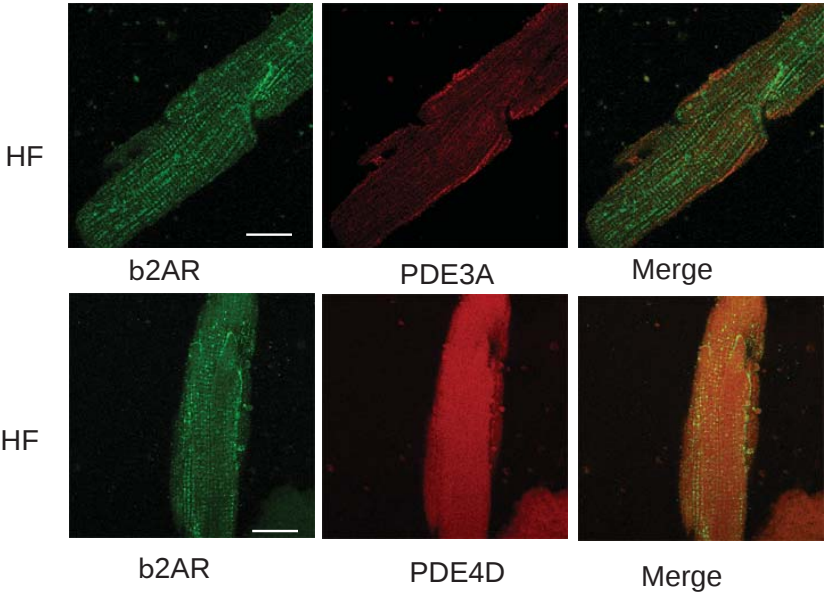


Supplemental Figure IV Continued

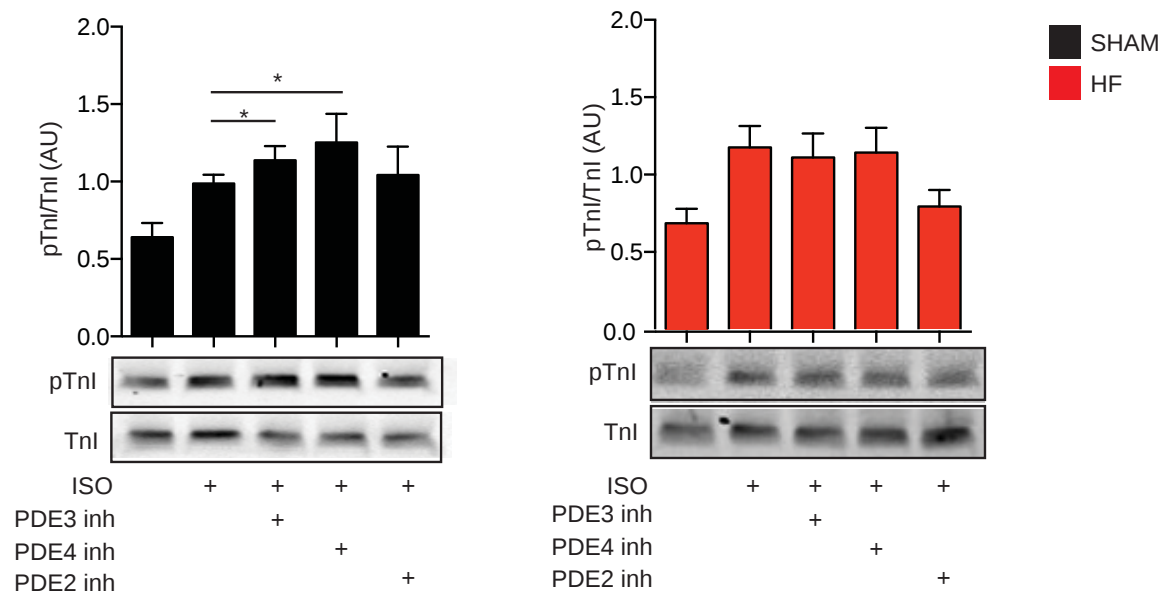
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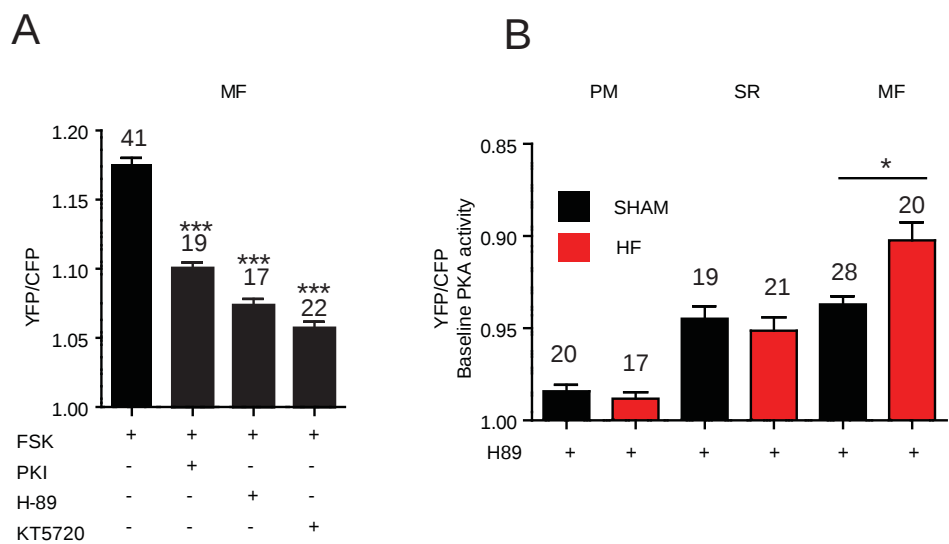
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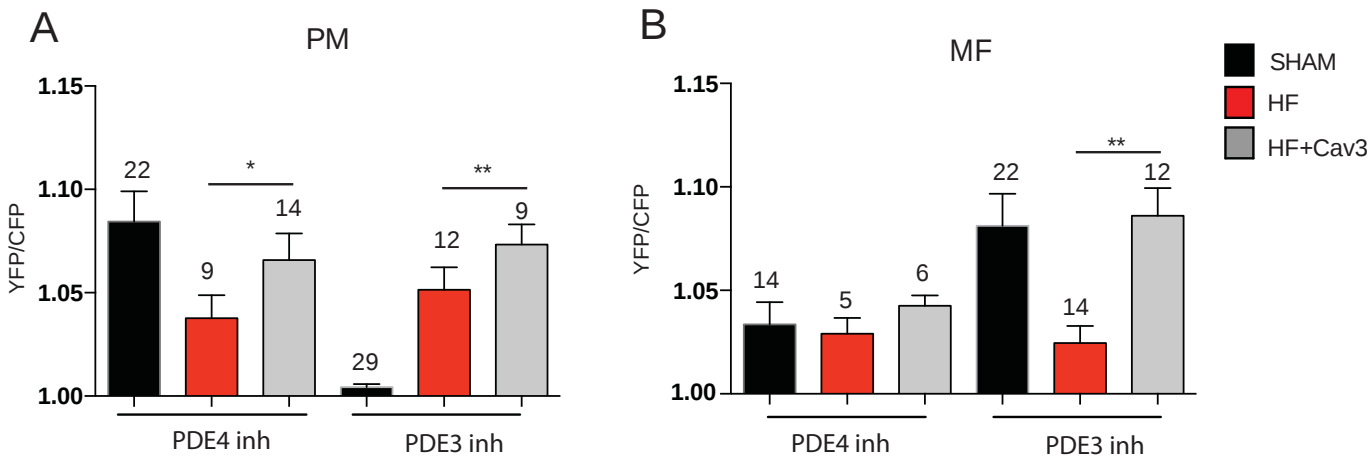
Supplemental Figure V



Supplemental Figure VI



Supplemental Figure VII



Supplemental Figure VIII

