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β -adrenergic receptor stimulation and activation of PKA protect against α_1 -adrenergic mediated phosphorylation of PKD and HDAC5

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Abstract

Introduction—Chronic activation of the β_1 -adrenergic receptor (β_1 -AR) signaling can have deleterious effects on the heart, and animal models over-expressing β_1 -ARs develop a dilated cardiomyopathy and heart failure. In the classical β -AR pathway, receptor occupancy by an agonist results in increased cyclic AMP (cAMP) levels and activation of protein kinase A (PKA). However, the role of PKA dependent signaling in the development and progression of cardiomyopathies and heart failure is controversial because β -AR signal transduction is generally desensitized in the failing heart, and PKA activity is not increased.

Methods and Results—Neonatal Rat Ventricular Myocytes (*NRVMs*) were acutely (15 minutes) or chronically (48 hours) treated with isoproterenol, and phosphorylation of protein kinase D (*PKD*) and histone deacetylase 5 (*HDAC5*) was measured. Acute β_1 -AR stimulation or expression of constitutively active (*CA*)-PKA reduced α_1 -adrenergic-mediated phosphorylation of HDAC5 and PKD by activation of a phosphatase. Over-expression of CA-PKA also reduced α_1 -adrenergic-mediated increased expression of contractile protein fetal isoforms and promoted repression of adult isoforms, but had no effect on α_1 -adrenergic-mediated cellular hypertrophy.

Conclusions—These data indicate that the PKA dependent arm of β -AR signaling can be anti-hypertrophic and presumably beneficial, through dephosphorylation of PKD and HDAC5 and reduction of hypertrophic fetal isoform gene expression.

Keywords

Cardiac hypertrophy; fetal gene program; phosphatase; PP2A

INTRODUCTION

While acute stimulation of cardiac β_1 -adrenergic receptors (β_1 -ARs) has been shown to have beneficial effects on myocardial performance, prolonged β_1 -AR activation ultimately

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impairs cardiac function (1-3). Along with other adverse biologic effects, chronic β_1 -AR activation leads to activation of a hypertrophic fetal gene program (FGP), resulting in increases in fetal isoforms such as β -myosin heavy chain (β MyHC), skeletal α -actin and A- or B- natriuretic peptide, and decreases in adult isoforms such as α MyHC and sarcoplasmic reticulum (SR)-associated Ca^{2+} ATPase (SERCA) (4-6). The remodeled/hypertrophied failing human heart exhibits induction of this fetal program of hypertrophic gene expression, and β_1 -receptor blockade is associated with reversion of gene expression towards an adult pattern in conjunction with reverse remodeling (3). In cultured rat ventricular cardiac myocytes β_1 -AR induction of the fetal gene program is mediated through Ca^{2+} -calmodulin dependent kinase II (CaMKII) signaling, and is independent of PKA (4).

In the classical description of the β_1 -AR signaling pathway, β_1 -AR stimulation activates protein kinase A (PKA) via an increase in adenylyl cyclase generation of cAMP. PKA then phosphorylates several downstream targets, including L-type Ca^{2+} channel (LTCC), phospholamban, troponin I and myosin binding protein C, leading to a wide range of effects on cellular calcium handling and contractility. LTCC phosphorylation increases Ca^{2+} influx; phospholamban inactivation by phosphorylation derepresses SERCA and increases SR Ca^{2+} reuptake as well as subsequent Ca^{2+} release; and troponin I and myosin binding protein C phosphorylation increase relaxation and contraction (reviewed in (7)). The immediate result of PKA-dependent phosphorylation of these effectors is increased cardiac myocyte contractile function and myocardial systolic and diastolic performance.

The role of PKA in altered myocardial β_1 -AR signaling in heart failure is controversial. β -AR signal transduction is generally desensitized in the failing human heart, and PKA activity (8) is not increased. Indeed, cAMP levels are actually decreased (9) in human failing ventricular myocardium, and phosphorylation of the PKA target phospholamban is also decreased (10). Desensitization and down-regulation of β_1 -ARs and up-regulation of $G_{\alpha i}$ thus result in attenuated cAMP signaling and a decreased response to β -adrenergic stimulation of contractility. However, there is evidence for PKA mediated hyperphosphorylation of ryanodine receptors (RyR) that may contribute to SR Ca^{2+} leak in the failing heart (11, 12), while other studies suggest that CaMKII is the kinase responsible for the detrimental effects of RyR phosphorylation in the failing heart (13, 14). In addition, unlike PKA dependent signaling, CaMKII signaling is maintained in the failing heart, through up-regulation of CaMKII activity (8, 15). Taken together, the data support a general hypothesis that chronic adrenergic activation in the failing heart results in adverse biologic effects mediated by CaMKII signaling, along with a loss in potential beneficial effects resulting from down-regulation of PKA dependent signaling.

In the work presented here we further investigate the role of PKA in the induction of hypertrophy in cardiac myocytes. The ability of PKA to block α_1 -adrenergic signaling was recently demonstrated by Haworth *et al* (16, 17) who showed that PKA activation by agents that increase cAMP reduces endothelin-1 (ET-1)- and phenylephrine (PE)-mediated phosphorylation of PKD, a known mediator of cardiac hypertrophy (16, 17). PKD and its upstream activator Protein Kinase C (PKC) have both been shown to phosphorylate Class II histone deacetylases (HDACs). HDACs act by deacetylating histones. Histone deacetylation results in a tighter interaction of the histones with genomic DNA, limiting the access of transcription factors to gene promoters and causing localized transcriptional silencing (18). In response to hypertrophic agonists such as ET-1 and phenylephrine, Class II HDACs are phosphorylated, resulting in their nuclear export to the cytoplasm and the activation of the hypertrophy-related fetal gene program. Here we show that acute β_1 -AR signaling resulting in activation of PKA blocks α_1 -AR-mediated phosphorylation of PKD and HDAC5, and inhibits expression of fetal hypertrophic genes. However, PKA over-expression results in down-regulation of the adult isoforms α MyHC and SERCA. These observations set the

stage for a potential anti-hypertrophic strategy involving selective PKA activation, including that delivered through β_1 AR stimulation or other agents that increase cAMP, combined with inhibition of CaMKII signaling.

METHODS

Materials

Isoproterenol (Sigma) was dissolved in H₂O containing 10⁻³M ascorbic acid and used at 10⁻⁷M final concentration. Additional reagents were added to the cells with the following final concentrations: 10⁻⁵M final concentration of phenylephrine (Sigma), 10⁻⁷M final concentration of okadaic acid (Biomol), 200nM CGP 20712A20712a (Tocris), 300nM ICI 118551 118551 (Tocris).

Antibodies

P-HDAC5 S498 was a gift from Dr. Tim McKinsey (University of Colorado, Denver), P-PKD, Ser-916 (2051) was purchased from Cell Signaling. Calnexin (4731) antibody was purchased from Sigma. PKA α antibody was purchased from Santa Cruz Biotechnology (sc-903). The HRP (115-035-146) anti-mouse and anti-rabbit were purchased from Jackson Laboratories.

DNA constructs

The CA-PKA construct was a gift of Dr. Eric Olson (University of Texas, Southwestern Medical Center).

Cell Culture and Cardiomyocyte transfection

Neonatal rat ventricular myocytes (NRVMs) were prepared according to the method described in Waspé *et al* (19) and plated in 0.1% gelatin coated plates. Cardiac myocyte transfections were done using the nucleofaction protocol (Amaxa, Inc). This methodology results in ~50% transfection efficiency (20). Briefly, 2 \times 10⁶ cells were transfected with 4 μ g of plasmid DNA according to manufacturer's conditions. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

Immunofluorescence and cell size measurements

Cell size was measured using Image J software (NIH) as previously described (21). Immunofluorescence was performed essentially as described (20). Images were captured at a 60X magnification with a fluorescence microscope Zeiss LSM 510 META on Axiovert 200M equipped with Zeiss Axiovision LE imaging software.

Real Time PCR

Total RNA was extracted by TRIZol (Invitrogen). 0.5 μ g of RNA were reverse transcribed into cDNA using I-script (Bio-Rad). Typically, 0.1 ng of cDNA, 12.5 nM of each primers and Power Syber Green PCR Master Mix (ABI) were used in the RT-PCR reactions. Reactions were performed using the ABI7300 system. The primers were described in (20). All RT-PCR experiments were repeated 5-7 times.

Western Blots

Western Blots were performed essentially as described (22). Antibodies were diluted 1:1000 in 1X TBS (20mM Tris 500mM NaCl pH 7.5) containing 3% BSA and 0.1% tween and

incubated with the blot overnight at 4°C. All western blot experiments were repeated 3-4 times.

Statistical Analysis

All analyses were performed using ANOVA with Bonferroni post-hoc test, with a $p < 0.05$ in a two-tailed distribution considered to be statistically significant.

RESULTS

β_1 -AR stimulation reduces phenylephrine (PE)-mediated increase in phosphorylation of PKD and HDAC5

Given that PKA is activated in response to β_1 -AR stimulation and that PKA activation has recently been shown to block PKD phosphorylation (16, 17), we hypothesized that a β_1 -AR agonist would act to reduce α_1 -adrenergic-mediated phosphorylation of PKD. In order to test this hypothesis we treated NRVMs for 15 minutes with the β -AR agonist Isoproterenol (ISO) before adding the α_1 -AR agonist PE. 15 minutes post PE treatment, NRVMs were harvested and PKD phosphorylation was analyzed by Western Blot. As shown in Figure 1A, ISO treatment significantly reduced PE-mediated phosphorylation of PKD, suggesting that β_1 -AR stimulation acted to protect the cells from α_1 -AR-mediated phosphorylation of PKD/HDAC5.

The protective effect of β_1 -AR stimulation also affected HDAC5 phosphorylation, a downstream target of PKD. As shown in Figure 1C, PE-mediated increases in phospho-HDAC5 were blunted by pretreatment of NRVMs with ISO. To test if this effect was mediated by β_1 -AR or β_2 -AR cells were pre-treated with the receptors specific blockers CGP20712a or ICI118551. As shown in Figure 2A and 2B, decreased phosphorylation of HDAC5 is mediated by β_1 -AR and not β_2 -AR.

PKA activity reduces PE-mediated increase in phosphorylation of PKD and HDAC5

The ability of ISO treatment to reduce α_1 -AR signaling suggests that PKA activation, a downstream effect of β_1 -AR stimulation, mediates this response. If so, it would be expected that increased PKA activity without β_1 -AR stimulation would have a similar effect on α_1 -AR signaling. In order to increase PKA activity in the absence of ISO treatment, NRVMs were transfected with a DNA construct that over-expresses a constitutively active form of PKA (CA-PKA). As shown in Figure 3A, cells transfected with the CA-PKA construct exhibited increased levels of PKA (Figure 3E), and a significant reduction in PE-related phosphorylation of PKD. Similarly, phosphorylation of HDAC5 was reduced when compared to cells transfected with the control vector (Figure 3B). These data suggest that PKA activation is responsible for β_1 -AR-mediated effects on PKD and HDAC5 phosphorylation.

PKA activity reduces PE-mediated increase of hypertrophic fetal genes

Since HDAC5 is a known transcriptional repressor of genes related to the hypertrophic Fetal Gene Program (FGP), over-expression of CA-PKA and the subsequent protection against PE-mediated phosphorylation of HDAC5 would be expected to attenuate the induction of the FGP in NRVMs treated with PE. In order to test this hypothesis, NRVMs were transfected with the CA-PKA plasmid and 24 hours later treated with PE for an additional 24 hours, with gene expression analyzed by RT-PCR. mRNA abundance analysis showed that CA-PKA significantly reduces PE-mediated increased expression of the FGP markers B-type Natriuretic Peptide (BNP), skeletal α -actin, and β -Myosin Heavy Chain (β MyHC) (Figure 4). Transfection with CA-PKA alone (without PE treatment) appeared to have no effect on these FGP markers. Together, these data further corroborate the protective effects

of PKA in the heart in that PKA activity prevents the induction of hypertrophy-related genes in response to α_1 -AR signaling.

PKA-mediated dephosphorylation of PKD and HDAC5 is likely mediated by PP2A

A possible explanation for the β_1 -AR-mediated effects on PKD and HDAC5 is that PKA activates a phosphatase that promotes dephosphorylation of PKD and HDAC5. PKA has the ability to activate Phosphatase 2A (PP2A) (23). If PP2A plays a role in the dephosphorylation of PKD or HDAC5, PP2A inhibition would be expected to prevent ISO-mediated dephosphorylation of PKD/HDAC5. In order to determine whether PP2A acts to dephosphorylate PKD/HDAC5, NRVMs were treated with 1nM of okadaic acid (a concentration known to inhibit PP2A (24)) before treatment with ISO. As shown in Figure 5, okadaic acid treatment reduces the protective effect of β_1 -AR stimulation on PE-mediated increases in PKD and HDAC5 phosphorylation.

PKA activity down-regulates “adult” contractile protein genes

Although acute β_1 -AR stimulation appears to have a protective effect on NRVMs, chronic β_1 -AR activation has been linked to the development of myocardial disease and progression of heart failure. We have previously shown that chronic β_1 -AR stimulation results in activation of the fetal gene program including down-regulation of α MyHC and SERCA (4). A potential explanation of the adverse effect of chronic adrenergic stimulation lies in the expression of “adult” contractile protein/ Ca^{2+} handling genes whose levels are decreased in pathological states. As shown in Figure 6, transfection of CA-PKA into NRVMs for 24 hours results in decreased expression of two critical adult genes, α MyHC and SERCA. Whereas CA-PKA transfection had no effect on “fetal” genes in NRVMs that were not treated with PE (Figure 4), CA-PKA transfection alone acted to reduce α -MyHC and SERCA levels. As expected, α -MyHC and SERCA levels were also decreased by treatment with PE. Unlike the fetal genes, however, transfection by CA-PKA did not act to block PE-mediated changes. Instead, CA-PKA over-expression and PE treatment had additive effects on the down-regulation of adult genes, suggesting that the regulation of fetal and adult gene expression is mediated by different pathways.

PKA activity does not prevent cellular hypertrophy

In order to test if the hypertrophic and gene expression changes are mediated by similar pathways we determined cell size in NRVMs expressing CA-PKA and treated with PE. As shown in Figure 7, expression of CA-PKA does not prevent PE-mediated increase in cell size. This result suggests that different signals are involved in promoting changes in gene expression and cellular hypertrophy, and that CA-PKA does not have an effect on PE-mediated cellular hypertrophy.

DISCUSSION

Activation of β_1 -AR signaling in conditions of stress (such as exercise, traumatic injury and blood loss) results in an elevation of cardiac output that is necessary to meet increased circulatory demand. However, chronic activation of β_1 -AR signaling is maladaptive and contributes to the progressive natural history of heart failure (25). Recent work by Wang *et al* (26) demonstrated that acute (10 minutes) β -AR signaling activates PKA, while chronic (24 hours) stimulation results in desensitization of the PKA pathway and increased sensitization of the CaMKII pathway. In our previous work we showed that chronic β_1 -AR stimulation leads to changes in gene expression and cell size that are associated with a pathologic response dependent on CaMK but not PKA (4). We now show that PKA activation can be protective against the induction of hypertrophy-associated gene expression in an acute setting. PKA activation likely results in activation of a phosphatase that then

promotes dephosphorylation of PKD and HDAC5 and reduces PE-mediated phosphorylation of these proteins in response to acute stimulation (Figure 8). Although not shown, NRVMs in culture for 48 h results in HDAC5 phosphorylation independent of PE treatment which preclude long term studies of the effects of PKA on PKD and HDAC5 phosphorylation.

Nuclear export of HDACs results in transcription de-repression and activation of gene expression (27). Here we show that β_1 -AR stimulation reduces PE-mediated phosphorylation of PKD and the Class II HDAC, HDAC5, in NRVMs. This effect is likely a result of β_1 -AR-mediated activation of PKA since transfection with a CA-PKA construct also reduces PE-mediated phosphorylation of PKD and HDAC5. In addition, CA-PKA reduces PE-mediated activation of the hypertrophic fetal gene program, blocking the up-regulation of BNP, skeletal α -actin, and β -MyHC expression, suggesting that PKA mitigates the deleterious effects of α_1 -adrenergic signaling. CA-PKA expression in NRVMs also results in down-regulation of the adult isoforms α -MyHC and SERCA, however, indicating that more chronic PKA activation does have negative effects on cardiac myocyte phenotype. Our results suggest that acute activation of the β_1 -AR can protect against pathologic changes in gene expression through dephosphorylation of PKD, but that chronic activation of PKA is likely detrimental due to down-regulation of α -MyHC and SERCA. The downstream signaling steps that account for the differences between acute and chronic PKA mediated signaling of components of the hypertrophic fetal gene program will need to be defined by additional studies. However, PKA activation of PP2A may be involved in the acute changes. We have previously shown that in H9C2 cells HDAC5 dephosphorylation is mediated by PP2A (28). Another study has shown that PP2A is involved in nuclear import of HDAC5 in endothelial cells (29). Here we show that inhibition of PP2A with okadaic acid blunted the ISO effect on dephosphorylation of PKD or HDAC5. These results suggest that PP2A is involved in the dephosphorylating PKD or HDAC5 in response to β -adrenergic receptor activation.

Recently, another study showed that PKA directly phosphorylates HDAC5 and prevents its translocation to the cytoplasm (30). In this study the authors show that PKA phosphorylation of HDAC5, through cAMP treatment, prevents PE-mediated increase in the expression of fetal isoforms. The authors did not analyze its effect on α MyHC and SERCA expression. Furthermore, the authors showed that cAMP treatment prevented PE-mediated cellular hypertrophy. In our study, CA-PKA did not prevent cellular hypertrophy. It is possible that this effect is mediated by PKA-independent cAMP-mediated activation of EPAC. EPAC has been shown to have both a hypertrophic effect and to prevent against hypertrophy (31), and at this time, it is unclear if the differences observed upon cAMP treatment and CA-PKA expression are due to other cAMP-activated proteins.

In this work we analyzed the effect of PKA activation in pathologic gene program. We demonstrated that acute (15 minutes) β_1 -AR stimulation through activation of PKA can protect cardiomyocytes against phosphorylation of proteins involved in promoting changes in gene expression that are associated with pathologic hypertrophy and heart failure. However, chronic (24 hours) PKA activation leads to down-regulation of α MyHC and SERCA, which has been shown to be detrimental to myocardial function. Interestingly, expression of CA-PKA did not block hypertrophic growth in response to PE treatment. This result suggests that independent pathways regulate expression of the fetal and adult isoforms and cell growth, and that HDAC5-mediated gene transcription repression primarily regulates expression of the fetal isoforms. Furthermore, our results underscore the importance of understanding the function of different components of signaling pathways in order to develop therapies that affect specific effects of deregulated pathways.

A limitation of this study is the use of neonatal cardiac myocytes. However, this is an established model to understand the pathways that respond to β -AR stimulation. Moreover, β -AR stimulation of adult cardiac myocytes does not result in changes in the hypertrophic gene program making it an unsuitable model to study pathways involved in changes in gene expression (data not shown). Studies in an animal model of CaMKII inhibition and β -AR stimulation are currently underway in our laboratory, and will properly address the effects of PKA activation in the setting of CaMKII inhibition in an in vivo model.

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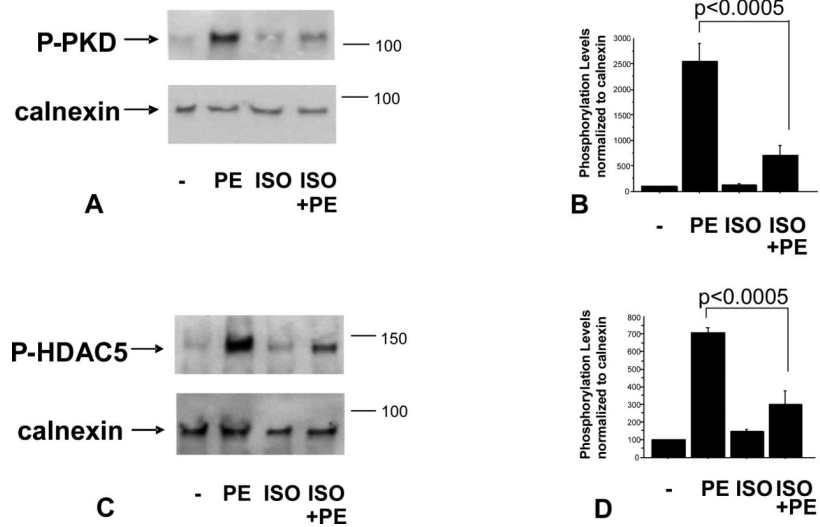


Figure 1. PE-mediated PKD (A, B) and HDAC5 (C, D) phosphorylation is reduced by ISO treatment. Cells were treated with 10^{-7} M isoproterenol (ISO) for 15 minutes followed by 15 minutes (A) or 1 hour (C) 10^{-5} M phenylephrine (PE) treatment. Phosphorylation levels were determined by western blot using PKD or HDAC5 phospho-specific antibody. Quantification of western blots is shown (B, D).

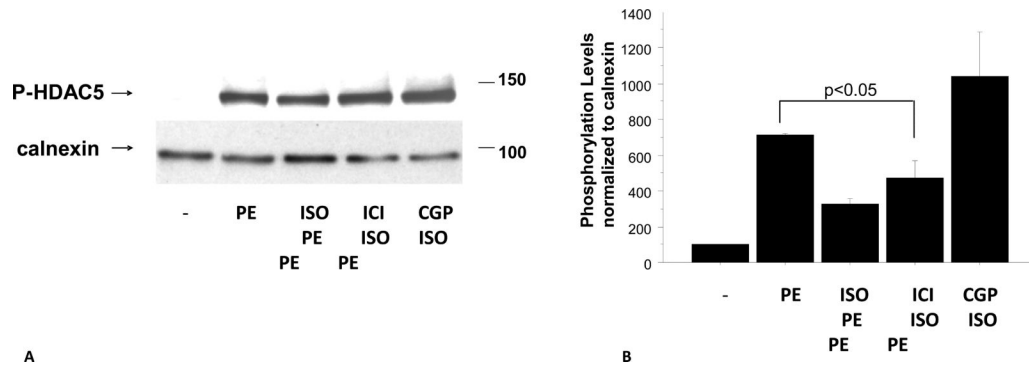


Figure 2.

ISO effect on HDAC5 phosphorylation is mediated by β_1 -AR and not β_2 -AR (A, B). Cells were treated with 200nM of the β_1 -AR CGP 20712A20712a or 300nM of the β_2 -AR ICI 118551 118551 (Tocris) followed by 10^{-7} M isoproterenol (ISO) for 15 minutes and 1 hour 10^{-5} M phenylephrine (PE) treatment. Phosphorylation levels were determined by western blot using HDAC5 phospho-specific antibody. Quantification of western blots is shown (B).

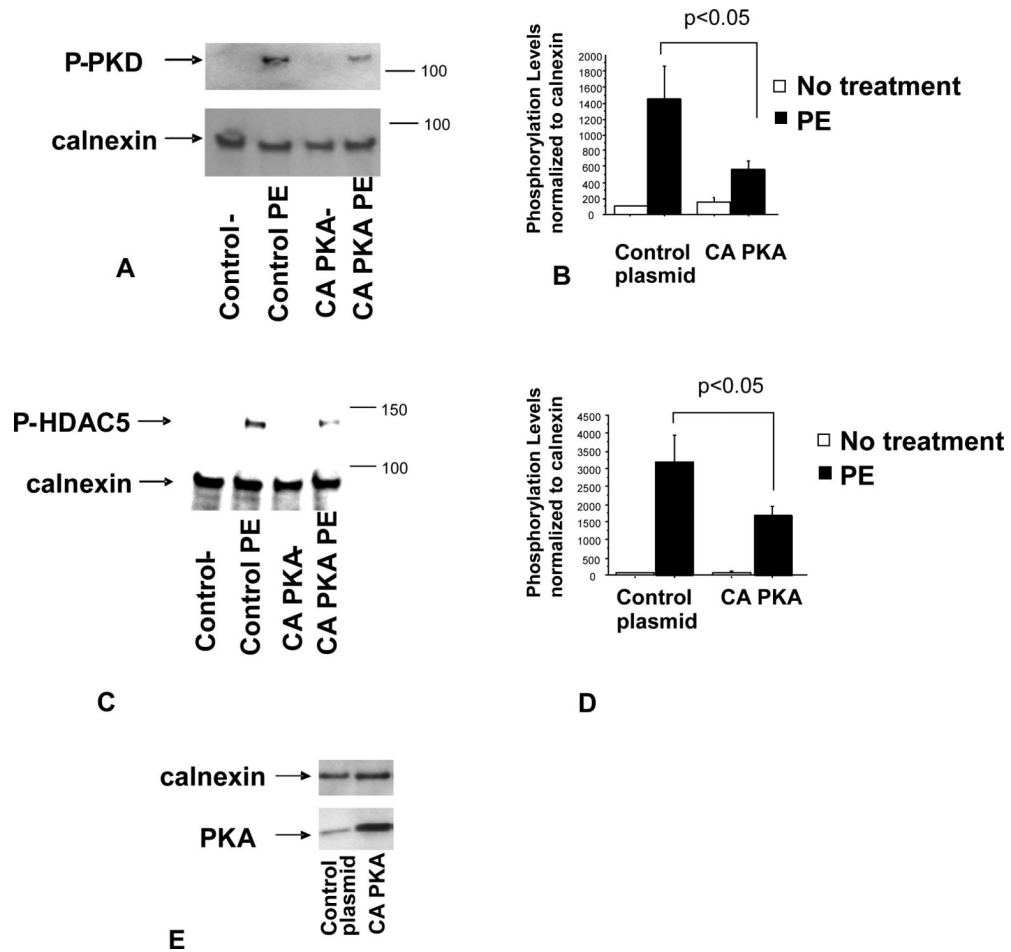


Figure 3. PE-mediated PKD (A, B) and HDAC5 (C, D) phosphorylation is reduced by overexpression of CA-PKA construct. Cells were transfected with a CA-PKA or a control plasmid and treated with 10^{-5} M PE for 15 minutes (A) or 1 hour (C) 24 hours after transfection. Phosphorylation levels were determined by western blot using PKD or HDAC5 phospho-specific antibody. Quantification of western blots is shown (B, D). (E) PKA levels are increased in response to transfection of CA-PKA construct. Cells were transfected using the Amaxa system and PKA levels were measured by western blot.

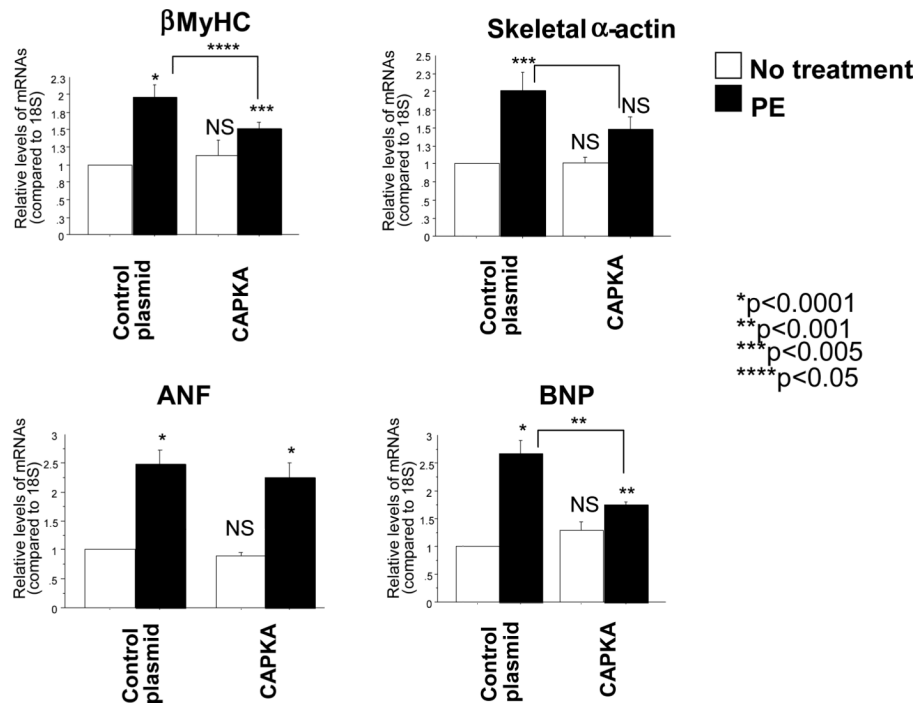


Figure 4. Over-expression of constitutively active PKA reduces PE-mediated increased gene expression of BNP, β MyHC and skeletal α -actin. Cells were transfected with a CA-PKA construct and treated with 10⁻⁵M PE 24 hours after transfection. RNA was extracted 48 hours after treatment and gene expression was analyzed by RT-PCR.

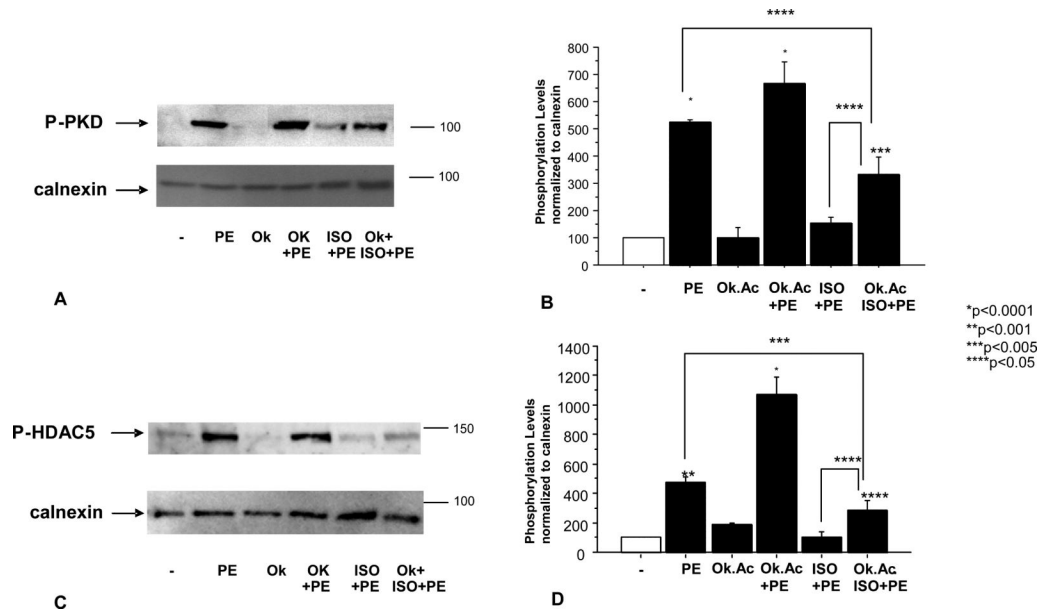


Figure 5. PP2A inhibition reduces ISO-mediated dephosphorylation of PKD (A) or HDAC5 (C). Cells were treated with 100 nM okadaic acid (Ok) 30 minutes prior to 10^{-7} M ISO treatment. Phosphorylation levels were determined by western blot. Treatment with Ok did not affect PKD and HDAC5 phosphorylation levels and are is represented in the graph.

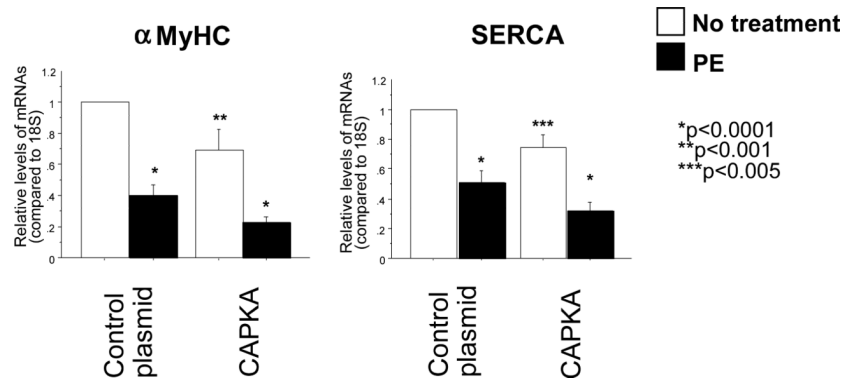


Figure 6. Over-expression of constitutively active PKA decreases expression of α MyHC and SERCA gene expression. Cells were transfected with a CA-PKA construct and treated with 10^{-5} M PE 24 hours after transfection. RNA was extracted 48 hours after treatment and gene expression was analyzed by RT-PCR.

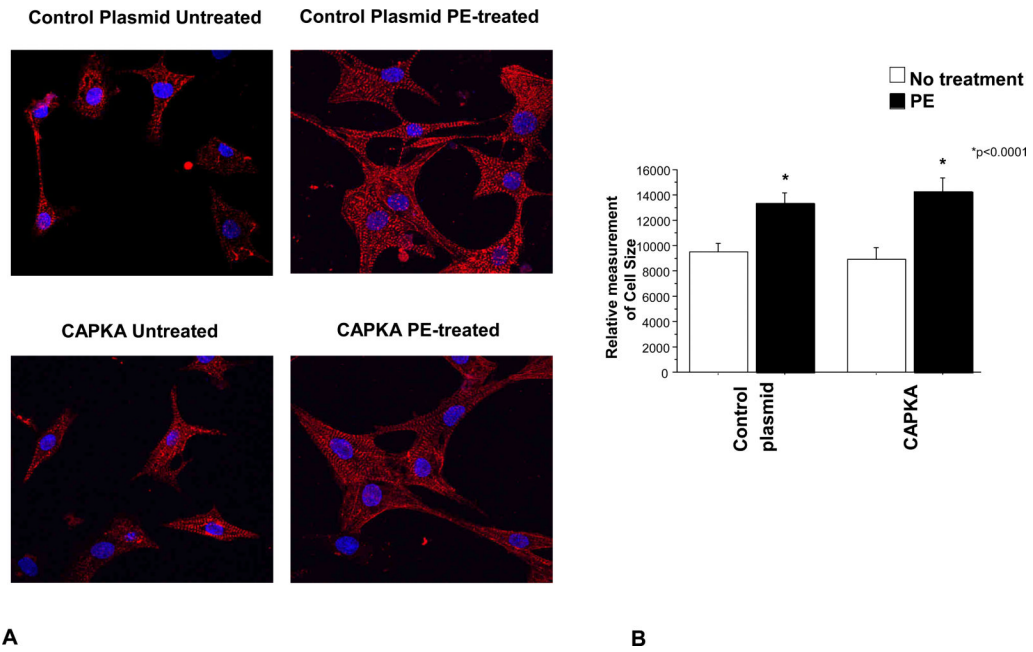


Figure 7. Over-expression of constitutively active PKA does not affect PE-mediated cellular hypertrophy. Cells were transfected with a CA-PKA construct and treated with 10^{-5} M PE 24 hours after transfection. Myocytes were immunostained with α -actinin and cell size was measured using ImageJ. A total of 55 cells were measured from 4 separate experiments.

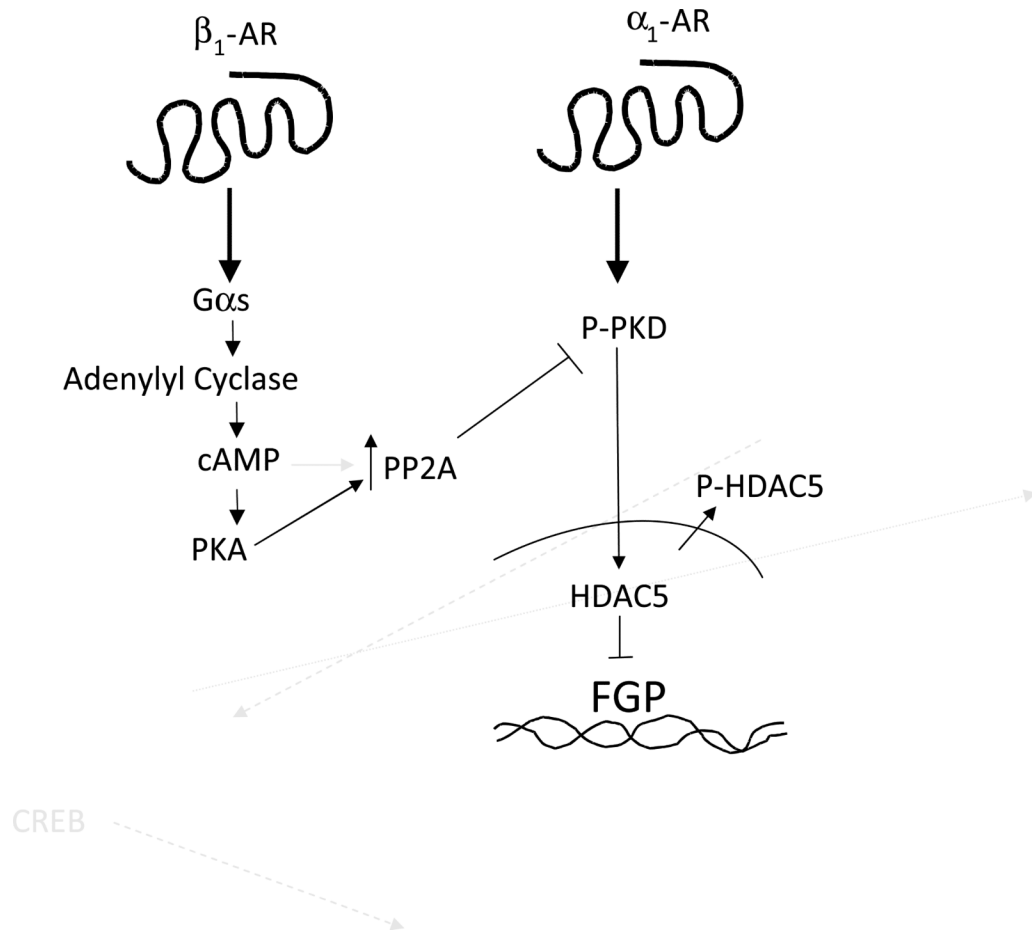


Figure 8. Schematic representation of the proposed regulatory pathway. Acute stimulation of the β_1 -AR results in activation of PKA and PP2A. This in turn prevents α_1 -AR-mediated phosphorylation of PKD and HDAC5 and gene expression activation of fetal isoforms.