Cardiotoxic and Cardioprotective Features of Chronic β-Adrenergic Signaling

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Rationale: In the failing heart, persistent β-adrenergic receptor activation is thought to induce myocyte death by protein kinase A (PKA)-dependent and PKA-independent activation of calcium/calmodulin-dependent kinase II. β-adrenergic signaling pathways also are capable of activating cardioprotective mechanisms.

Objective: This study used a novel PKA inhibitor peptide to inhibit PKA activity to test the hypothesis that β-adrenergic receptor signaling causes cell death through PKA-dependent pathways and cardioprotection through PKA-independent pathways.

Methods and Results: In PKA inhibitor peptide transgenic mice, chronic isoproterenol failed to induce cardiac hypertrophy, fibrosis, and myocyte apoptosis, and decreased cardiac function. In cultured adult feline ventricular myocytes, PKA inhibition protected myocytes from death induced by β1-adrenergic receptor agonists by preventing cytosolic and sarcoplasmic reticulum Ca2+ overload and calcium/calmodulin-dependent kinase II activation. PKA inhibition revealed a cardioprotective role of β-adrenergic signaling via cAMP/exchange protein directly activated by cAMP (EPAC) and extracellular signal-regulated kinase pathway. Selective PKA inhibition causes protection in the heart after myocardial infarction that was superior to β-blocker therapy.

Conclusions: These results suggest that selective block of PKA could be a novel heart failure therapy. (Circ Res. 2013;112:498-509.)

Key Words: apoptosis ■ Ca2+/calmodulin-dependent protein kinase II ■ extracellular signal-regulated kinases 1/2 ■ exchange protein directly activated by cAMP ■ cAMP ■ protein kinase A inhibition

Congestive heart failure (CHF) affects 5 million people in the United States, causing high morbidity and mortality rates.1 The poor pump function of the failing heart induces chronic activation of neuroendocrine systems that supports cardiac performance but also can activate death signaling to cause CHF progression.

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Persistent activation of the sympathoadrenergic system in CHF can cause adverse cardiac remodeling, cardiac myocyte death, and fibrosis replacement.2 The hypothesis of this study is that β-adrenergic-mediated myocyte death requires PKA activation and subsequently enhanced Ca2+ signaling, but is independent of EPAC. To test this idea, we designed a PKA-specific inhibition gene (a fusion gene containing the nucleotide sequence coding the amino acids 1–25 of PKA inhibitor peptide [PKI]-α and green fluorescent protein [GFP, PKI-GFP]). PKI-GFP was expressed in mouse heart or in cultured adult feline ventricular myocytes (AFVMs). Our major findings are: (1) β-agonists activated both PKA and EPAC, and PKI-GFP inhibited only PKA-mediated of β-adrenergic-induced myocyte apoptosis by altering Ca2+ regulation,5,5 whereas others have suggested that Ca2+/calmodulin-dependent kinase II (CaMKII) can mediate β-adrenergic-induced myocyte death through a PKA-independent process.6–10 A cAMP sensor, exchange protein directly activated by cAMP (EPAC), is expressed in the heart and has been suggested to activate CaMKII independent of PKA.6–8

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Nonstandard Abbreviations and Acronyms

AFVM = adult feline ventricular myocyte
β-AR = β-adrenergic receptor
CaMKII = Ca2+/calmodulin-dependent kinase II
CHF = congestive heart failure
DTG = double-transgenic
EPAC = exchange protein directly activated by cAMP
GFP = green fluorescent protein
ISO = isoproterenol
LTCC = L-type calcium channel
MI = myocardial infarction
PKA = protein kinase A
PKI = PKA inhibitor peptide
SR = sarcoplasmic reticulum
VM = ventricular myocyte

Methods

A DNA oligo corresponding to the coding sequence for amino acids 1 to 25 of mouse Pkia (mouse Entrez gene ID 18767) was synthesized and subcloned into a plasmid to make a PKI-GFP fusion gene. Amino acids 1 to 25 of PKI have the PKA inhibitory domain but not the nuclear export signal.12 An adenovirus containing the fusion gene and a transgenic mouse line expressing this fusion gene (Online Figure I) were established.13 Doxycycline-containing (625 ppm) chow was offered to breeding animals and preweaned pups. Transgenic and littermate control animals were used at the age of 4 months (Online Figure I). To test β-adrenergic overstimulation on cardiac myocyte death, acute isoproterenol (ISO; 60 mg/kg) or chronic ISO (60 mg/kg per day for 3 weeks) was applied.14 Echocardiography, cardiac morphology, gravimetric measurements, tissue histology, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining were performed at the end of 3 weeks.14 To explore PKA-dependent and PKA-independent mechanisms of myocyte apoptosis induced by β-AR signaling, AFVMs were isolated, cultured, and infected with AdGFP (control) or AdPKI-GFP.15 The inhibition of PKA by PKI-GFP was determined with a nonradioactive PKA activity kit (Assay Design), and cAMP production on ISO stimulation was determined with [3H]-adenine and radioactivity incorporation into newly synthesized cAMP. Myocyte death was determined by trypan blue staining, rod/ball ratio counting, TUNEL, and fluorochrome inhibitor of caspases staining. Myocyte contractions and intracellular Ca2+ transients, Ca2+ currents, and sarcoplasmic reticulum (SR) Ca2+ content were measured as previously described.14 To determine the activity of PKA and CaMKII, phospholamban (PLB) phosphorylation at Ser16 and Thr17 and CaMKII phosphorylation at Thr286 were determined with Western blot. Western blot also was used to determine activated Rap1 that bound to GTP (Rap1GTP), ERK, and pERK. To determine the effect of β-blockade on the protection of PKI in post-MI mice, littermate control and PKI double-transgenic (DTG) mice were injected with saline or metoprolol (20 mg/kg body weight per day) daily15 for 4 weeks. Cardiac function was followed weekly with echocardiography. An expanded Methods can be found in the online-only Data Supplement.

Results

PKI-GFP Is Evenly Expressed in the Mouse Heart to Suppress PKA Activity

The PKI-GFP DTG mouse began to express PKI-GFP at 2 months and had stable expression by 4 months of age (Figure 1A). PKI-GFP was expressed in DTG mice but not in PKI-GFP single transgenic, tet-controlled transactivator only, and in wild-type mice (Figure 1B). Homogenous GFP fluorescence was seen in DTG hearts but not in PKI-GFP single transgenic hearts (Figure 1C). In isolated ventricular myocytes, the GFP fluorescence was evenly distributed in the whole cell (Figure 1D). There was little PKA activity at baseline in both control

Figure 1. Protein kinase A (PKA) inhibitor peptide (PKI)–green fluorescent protein (GFP) overexpression in mouse hearts inhibits PKA. A, The expression of PKI-GFP in double-transgenic (DTG) mice was stable at the age of 4 months. B, PKI-GFP was not expressed in ventricles of 4-month-old single-transgenic (STG) mice with PKI-GFP (no tet-controlled transactivator [tTA] or tTA only, or in wild-type mice. C, GFP expression is homogenous in the DTG hearts but not expressed in the PKI STG hearts, agreeing with the Western blot results. D, Confocal images of a live DTG ventricular myocyte. PKI-GFP is evenly distributed in isolated PKI-DTG myocytes. E, PKA activity without or with cAMP. PKA activity is inhibited by >90% in the crude extract from DTG hearts compared with control hearts. DAPI indicates 4',6-diamidino-2-phenylindole.
and DTG hearts. However, when PKA activity in crude cardiac tissue extract was measured in the presence of 1 μmol/L cAMP, PKA activity was almost completely inhibited in PKI transgenic samples (Figure 1E).

**PKI-GFP Prevents Cardiac Dysfunction and Myocyte Death Induced by ISO**

We first examined whether β-adrenergic-induced myocyte death was PKA-dependent (in vivo) by measuring the effect of ISO on myocyte apoptosis in the PKI-DTG mice. Chronic ISO infusion into PKI transgenic and control mice for 3 weeks induced cardiac hypertrophy and decreased cardiac function in control animals but not in PKI transgenic mice (Figure 2A and 2B). Chronic ISO infusion caused significant fibrosis in control cardiac tissue but not in PKI-DTG tissue (Figure 2C and 2D). There were significant increases in TUNEL + cardiac myocyte nuclei in control cardiac tissues but not in PKI-DTG tissue (Figure 2E). A single injection of ISO into PKI-DTG and control mice induced a higher percentage of TUNEL + cardiac myocytes in control myocardium than in PKI-DTG myocardium (Figure 2E).

**PKI-GFP Overexpression in Cultured AFVMs Prevents PKA Activation But Does Not Affect ISO-Induced cAMP Production**

PKI-GFP should blunt β-agonist-induced activation of PKA without altering cAMP production. To test this idea, AFVMs were infected with AdPKI-GFP or AdGFP. At 48 hours post infection, GFP and PKI-GFP were expressed (Figure 3A and 3B). Both PKI-GFP and GFP were evenly distributed through the cytoplasm of infected cells. The crude protein extract contained a low activity of PKA, which was not different between the groups; 1 μmol/L cAMP increased the PKA activity in the control group in the crude extract from AdGFP infected cells but not in the crude extract from AdPKI infected cells (Figure 3C). When these 2 groups of myocytes were exposed to 10 μmol/L ISO for 10 minutes in the presence of a phosphodiesterase (PDE) inhibitor, isobutylmethylxanthine, the total amount of cAMP produced was not different between groups (Figure 3D), showing no change of β-adrenergic signaling upstream of PKA.

**PKI Prevents Myocyte Apoptosis Induced by β-Adrenergic Agonists in AFVMs**

Excessive β-AR stimulation can lead to myocyte death via apoptosis.18 AdPKI-GFP–infected AFVMs (PKI-AFVMs) were exposed to β-AR agonists to induce myocyte death. PKI-AFVMs were completely protected from cell death induced by ISO (a nonselective β1-AR and β2-AR agonist; 10 μmol/L; Figure 4A and 4B). The survival of ventricular myocytes (VMs) exposed to 10 μmol/L ISO for 72 hours was increased when AdPKI-GFP multiplicity of infection was increased from 10 to 100 (Figure 4C). This protection effect was associated with reduced myocyte apoptosis measured by TUNEL and fluorochrome inhibitor of caspases (an index of caspase activity; Figure 4D). These results do not agree with a previous study showing that exogenous PKA inhibitors could not rescue rodent myocyte death induced by β-AR activation.19 This could be because of the fact that our studies were performed with AFVMs, which possess physiological properties similar to human ventricular myocytes and do not accumulate Ca2+ under unpaced culture condition.20 Rodent myocytes accumulate Ca2+, have SR Ca2+ overload in culture, and have a high rate of apoptosis without β-adrenergic agonists.20 Any residual unblocked PKA effect would enhance SR Ca2+ overload in rodents, although these effects are absent in AFVMs.

**β-Adrenergic Toxicity Is Mediated by β1-Adrenergic Receptor and Linked to cAMP/PKA Activation**

Dobutamine (a β1-AR agonist), ISO plus ICI 118551 (a β2-AR antagonist; 2 μmol/L), ISO plus CGP 20712A (a β1-AR antagonist; 6 μmol/L), fenoterol (a β2-AR agonist; 10 μmol/L), and forskolin (an adenylyl cyclase activator) were used to determine the effect of β1-AR vs β2-AR activation on myocyte death (Figure 4F). Dobutamine, ISO plus ICI, and forskolin induced myocyte death that was rescued by PKI-GFP. These results suggest that β1-AR activation and subsequent adenylyl cyclase and PKA activation are primarily responsible for

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**Figure 2.** Protein kinase A (PKA) inhibition abolishes myocardial remodeling induced by isoproterenol (ISO) infusion (3 weeks) in PKI transgenic (DTG) mice. PKI prevents cardiac hypertrophy (A, heart weight/body weight ratio), cardiac function decrease (ejection fraction, B), fibrosis (C and D), and myocyte apoptosis (E). Some hearts were treated with ISO injection and some hearts were treated with ISO minipump for 3 weeks. B, *P<0.05, control ISO 3 weeks vs control before ISO; #P<0.05, control ISO 3 weeks vs PKI ISO 3 weeks. The numbers in the bars of A, D, and E are numbers of animals.
myocyte death induced by ISO. In contrast, when myocytes were stimulated via β2-AR (ISO plus CGP or fenoterol), only a small fraction of myocytes died. PKI also blocked this proapoptotic effect. These results agree with those studies showing that β1-AR is the major mediator of β-adrenergic toxicity, whereas β2-AR activation leads to much less myocyte death.21

The new finding here is that the adenylyl cyclase/cAMP/PKA signaling pathway is the exclusive pathway mediating myocyte death, at least in cultured AFVMs.

**PKI Prevents Myocyte Apoptosis Induced by β-AR Stimulation by Reducing PKA-Mediated SR Ca2+ Overload**

β-adrenergic agonists enhance SR Ca2+ by augmenting Ca2+ influx through the L-type calcium channel (LTCC; I Ca-L) and by stimulating SR Ca2+ uptake through PLB phosphorylation.22 We14,15 and others19 have found that this can lead to SR Ca2+ overload, which can cause myocyte death both in vitro15 and in vivo.14 Exposure of AFVMs to ISO (10 μmol/L) for 1 hour increased the percentage of spontaneously contracting myocytes in GFP-VMs, indicating SR Ca2+ overload.15 PKI significantly decreased the percentage of spontaneously contracting myocytes under this condition (Figure 5A). The LTCC blocker, nifedipine (13 μmol/L), decreased myocyte death induced by ISO and offered no further protection than PKI, indicating that PKI protects myocytes by reducing the increases in LTCC activity caused by β-adrenergic stimulation. Intracellular Ca2+ buffering (1,2-bis[2-aminophenoxy]ethane-N,N,N',N'-tetraacetic acid tetrakis[acetoxymethyl ester]-AM; 10 μmol/L) completely blocked GFP-VM death. A specific sarco/endoplasmic reticulum Ca2+-ATPase inhibitor, thapsigargin (10 nmol/L),15 also largely blocked ISO-induced myocyte death and had no detrimental effects in PKI-VMs (Figure 5B).

The effects of PKI on ISO effects on I Ca-L, contraction, Ca2+ transients, and SR Ca2+ content were measured. PKI abolished the acute stimulatory effects of ISO on myocyte I Ca-L, fractional shortening, Ca2+ transients, and SR Ca2+ load (Figure 5C–5F).

A previous report suggests that chronic β-adrenergic stimulation can enhance Ca2+ handling in cultured myocytes independent of PKA but dependent on Ca2+/CaMKII.10 In our study, chronic ISO (10 μmol/L) exposure for 24 hours significantly increased I Ca-L, fractional shortening, Ca2+ transient, and SR Ca2+ content in GFP-VMs but not in PKI-VMs (Figure 5C–5F). The blunted response to ISO in PKI-VMs was not because of changes in the expression of Ca2+ handling proteins (α1c, ryanodine receptor 2, calsequestrin, total PLB, Na+/Ca2+ exchanger1, and sarco/endoplasmic reticulum Ca2+-ATPase; Figure 6 and Online Figure II). These results indicate that in AFVMs, the positive inotropic effect of chronic ISO stimulation is mediated by PKA and the protective effect of PKI against ISO-induced myocyte death is largely mediated by preventing cytotoxic and SR Ca2+ overload.

**ISO-Induced CaMKII Activation Is Downstream of PKA**

It has been shown that CaMKII plays a critical role in adrenergic19,23 and Ca2+ toxicity15 in cardiac myocytes. Previous studies have suggested that CaMKII can be activated independently of PKA during chronic β-adrenergic stimulation.6–8,10,19 A central role for CaMKII as the mediator of chronic ISO-induced myocyte death was found in our system, because KN93, a CaMKII inhibitor, rescued most GFP-VMs from death induced by ISO (Figure 6A). KN93 did not improve PKI-VM survival after ISO exposure.

It is clear that β-AR stimulation can activate CaMKII via PKA-dependent increases in I Ca-L and SR Ca2+.24 However, others suggest that CaMKII activation seen in myocytes chronically exposed to β-agonists is independent of PKA.7,8,10,19 We explored this issue by measuring PLB phosphorylation at Thr17 (a CaMKII-specific phosphorylation site) and CaMKII autophosphorylation at Thr286 in GFP-AFVMs and PKI-AFVMs. Myocytes were exposed to ISO for either 10 minutes or 24 hours. PKI inhibited phosphorylation of PLB at both Ser16 (PKA site) and Thr17 (a CaMKII-specific phosphorylation site) and PKI-AFVMs, without or with 1 μmol/L cAMP, cAMP production in AdGFP-, and AdPKI-infected cells is not different.
To rule out the possibility that PKI directly inhibits CaMKII, GFP-AFVMs and PKI-AFVMs were paced or coinfected with adenovirus containing LTCC β2a subunit, or were treated with an LTCC agonist (FPL 64176; 1 μmol/L) to activate CaMKII via directly increased cellular [Ca2+] without PKA activation. All these treatments induced PLB Thr17 phosphorylation in both GFP-VMs and PKI-VMs. FPL also enhanced CaMKII phosphorylation at Thr286 to the same extent in PKI and GFP-VMs. These data document that PKI does not inhibit CaMKII directly (Online Figure IIIA–IIID).

Previous studies suggest that increased intracellular cAMP activates EPAC to activate CaMKII independent of PKA. These studies used 8-cp-TOME, a so-called EPAC-specific activator, to activate EPAC. There is evidence that 8-cp-TOME may inhibit phosphodieseterase, leading to increased intracellular cAMP and, thus, PKA activation. 8-cp-TOME induced phosphorylation of PLB Ser16 (PKA site) and Thr17 (CaMKII site) in GFP-VMs in a dose-dependent manner. 8-cp-TOME could not activate PKA nor CaMKII in PKI-VMs (Online Figure IV).
Collectively, these results suggest that CaMKII activation induced by β-adrenergic stimulation is mediated by PKA in AFVMs. To determine whether the increase in Ca²⁺ influx promoted by PKA activation is responsible for Ca²⁺ overload, PKA inhibition significantly reduced the percentage of spontaneously contracting myocytes. In VMs not stimulated, there was no spontaneous contraction.

β-Agonists Induce Cardioprotection in PKI-Treated Myocytes by Activating EPAC

β-agonists increase cAMP that is able to activate EPAC. However, EPAC-specific effects on myocytes are difficult to evaluate because of parallel PKA activation. In our PKI-VMs, PKA activity is inhibited and this provides a system to evaluate whether EPAC regulates cell death signaling. EPAC, as a GTP exchange factor, activates Rap1-GTPase by catalyzing the formation of Rap1-GTP, which activates ERK. As predicted, both ISO and 8-cp-TOME exposure increased the amount of Rap1-GTP (Figure 7A), indicating EPAC activation. High extracellular Ca²⁺ ([Ca²⁺]₀ = 2.5 mmol/L) induced myocyte death at an equal rate in VMs infected with AdGFP and AdPKI, indicating high extracellular Ca²⁺-induced VM death is PKA-independent. The EPAC activator 8-cp-TOME (0.1 μmol/L, a concentration not activating PKA; Online Figure IV) did not have any effect on myocyte survival in both GFP-VMs and PKI-VMs.
in normal $[\text{Ca}^{2+}]_{o}$ (Figure 7B). 8-cp-TOME protected GFP-VMs and PKI-VMs from death induced by high $[\text{Ca}^{2+}]_{o}$, indicating EPAC offers protection on VMs. In GFP-VMs, forskolin and ISO increased Ca$^{2+}$-mediated myocyte death. In PKI-VMs, forskolin and ISO protected myocytes from Ca$^{2+}$-mediated death and the effect was equivalent to that of 8-cp-TOME in GFP-VMs (Figure 7E). These data show that β-adrenergic agonists activate proapoptotic signaling via PKA/CaMKII signaling and in parallel activate cardioprotective signaling via EPAC.

EPAC activation induces ERK phosphorylation and phosphorylated ERK protects various cells from death.19,26–29 ERK phosphorylation was increased in both GFP-VMs and PKI-VMs exposed to ISO and 8-cp-TOME (0.1 μmol/L, an EPAC-specific activator at low concentrations; Online Figure IV and Figure 7C and 7D), indicating that this process is PKA-independent (Figure 7B and 7C). To confirm that activated ERK is the cardioprotective mediator, an ERK inhibitor, PD 98059, was used to pretreat AFVMs before exposure to ISO or 8-cp-TOME and high extracellular Ca$^{2+}$. PD98059 almost completely abolished the protective effects of 8-cp-TOME in both GFP-VMs and PKI-VMs. The protective effect of ISO in PKI-VMs also was abolished by PD98059 (Figure 7E). These results strongly support the idea that ERK phosphorylation, downstream of EPAC activation, mediates the protective effects of adrenergic activation.

β-Blockade Eliminates the Protective Effect of β-Adrenergic Activation

The present results suggest that β1-AR agonists can induce cell death signaling through PKA and cardioprotective signaling through EPAC/ERK. β1-AR antagonist therapies could abolish both cardiotoxic and cardioprotective features of β1-AR activation. Selective block of PKA with PKI in cardiovascular disease could preserve cardioprotective aspects of
β1-AR signaling to provide benefit beyond β-AR antagonists. To test this idea MI was induced in wild-type and PKI-DTG mice, with and without concomitant β1-AR antagonist therapy. We used a β1-AR blocker, metoprolol, to induce similar reductions of PKA activity as our PKI-GFP gene does after MI (Figure 8A). MI caused a reduction in cardiac function in all animals and both β-blocker and PKI improved function. However, PKI-DTG animals had better cardiac function than β-blocker-treated animals. It seems that metoprolol at the dose used (20 mg/kg body weight per day) decreased but did not abolish the beneficial effects of PKI, suggesting that it blocked some but not all cardioprotective adrenergic signaling (Figure 8B).

**Discussion**

Common cardiovascular diseases, such as hypertension and ischemic heart disease, increase the contractile demand of the heart and the sympathoadrenergic system is activated to maintain basal cardiac output. Persistent sympathoadrenergic system activation is associated with myocyte death and cardiac decompensation, culminating in heart failure. How persistent activation of β-ARs induces myocyte death is not clearly defined and was the topic of this study. The specific roles of downstream β-AR signaling effectors (PKA, CaMKII, and EPAC) were examined. A novel PKA inhibition gene (PKI-GFP) was developed to inhibit PKA in vivo (transgenic mouse) and in vitro (cultured AFVMs infected with AdPKI-GFP). The new findings in this study are: (1) PKI-GFP can inhibit β-AR-mediated activation of PKA; (2) PKA inhibition with PKI reduces β-adrenergic agonist–induced myocyte death by preventing myocyte Ca2+ overload; (3) inhibition of PKA eliminates β-AR induced CaMKII activation; (4) β-AR-mediated increases in cAMP activates EPAC, and this exerts an ERK-dependent protective effect on myocyte death; and (5) PKI protects the heart after MI and a β1-AR antagonist, metoprolol, reduced the protection exerted by PKI. Collectively, these results show that β-AR signaling can induce both cell death (through PKA, CaMKII, and SR Ca2+ overload) and cardioprotection (through EPAC).
Figure 8. Metoprolol, a β1-blocker, reduces the protection of protein kinase A (PKA) inhibitor peptide (PKI) in mice with myocardial infarction (MI). A. Phospholamban (PLB) phosphorylation at the PKA site (Ser16) at 24 hours post-MI in mice. A dose of metoprolol that induced similar PKA inhibition (revealed by similar PLB phosphorylation at the Ser16 site) as PKI was used. B. Cardiac function (fractional shortening) in control and PKI double-transgenic (DTG) MI mice receiving saline or metoprolol treatment. C. Immunostaining of apoptotic nuclei (TUNEL, green), nuclei (DAPI, blue), and cell membrane (TRITC-conjugated lectin [TRITC-LECTIN], red) in infarcted zone at 2 days after coronary artery ligation. D, TUNEL rates in the infarcted zone in control and PKI-DTG mice receiving saline or metoprolol treatments.
PKA-Dependent Activation of CaMKII Is the Mediator of Myocyte Apoptosis Induced by β-Adrenergic Agonsists

Previous studies support the conflicting idea that CaMKII can be activated by either PKA-dependent or PKA-independent mechanisms after β-adrenergic stimulation. PKA-dependent activation of CaMKII is brought about by an increase in Ca²⁺ influx through the L-type Ca²⁺ channel after PKA-dependent phosphorylation. Recently, it has been reported that EPAC, a cAMP sensor, can activate CaMKII independently of PKA activation in cardiac myocytes. This is an important topic because CaMKII activation is required for myocyte apoptosis induced by β-adrenergic stimulation. Inhibition of CaMKII in vivo significantly reduced myocyte apoptosis after ISO stimulation, suggesting that CaMKII would be a useful therapeutic target in CHF. Our results show that CaMKII activation enhances the phosphorylation of CaMKII site (Thr17) on PLB in normal cardiac myocytes. In the presence of PKA inhibition, β-adrenergic stimulation had almost no effect on CaMKII autophosphorylation and PLB Thr17 phosphorylation. The present experiments show that when the β-adrenergic-induced increase in Ca²⁺ influx through the L-type Ca²⁺ channel is prevented by PKI or blocked by nifedipine, CaMKII activity was eliminated. These results suggest that at least in PKI mice and PKI-infected AFVMs, CaMKII activation is dependent on PKA-mediated increase in LTCC activity and subsequent increase in [Ca²⁺]. We also show that in the presence of PKA inhibition, β-AR agonists activate Rap1 downstream of EPAC activation, but CaMKII is not activated. These results show that EPAC does not cause CaMKII activation under our experimental conditions.

The present results contrast with those studies suggesting a PKA-independent, EPAC-mediated CaMKII activation during β-adrenergic stimulation. The basis for these disparate results is not clear but might be because of differences in species, experimental conditions (eg, concentrations of ISO and 8-cp-TOME), and methods to inhibit PKA. Studies suggesting EPAC-mediated CaMKII activation have used rodent myocytes in long-term culture. These preparations can be problematic because rodent myocytes accumulate Ca²⁺ when not paced. This results from their high intracellular Na⁺, which promotes Ca²⁺ entry via reverse mode of Na⁺/Ca²⁺ exchange. Therefore, cultured rodent myocytes have SR Ca²⁺ overload, as evidenced by the spontaneous SR Ca²⁺ sparks that are exhibited by these preparations. The persistently high intracellular Ca²⁺ in these cultured rodent myocytes could activate CaMKII. Our study used AFVMs, which, like humans, have lower intracellular Na⁺ and there is no Ca²⁺ accumulation or spontaneous SR Ca²⁺ release in long-term culture. The low basal Ca²⁺ state of AFVMs allowed us to dissect the role of PKA in mediating β-adrenergic-induced CaMKII activation. We also found that 8-cp-TOME can activate PKA possibly by indirectly inhibiting phosphodiesterase, as suggested by one previous study. The Ki values for PDE1B, PDE2, and PDE6 of 8-cp-TOME are 8.6 μmol/L, 15 μmol/L, and 3.5 μmol/L, respectively. Therefore, the interpretation of the results obtained with 10 μmol/L 8-cp-TOME should be cautious and adequate PKA inhibition should be ensured when evaluating EPAC effects with 8-cp-TOME. Our results clearly showed that in AFVMs, when PKA activity is adequately inhibited, 8-cp-TOME cannot activate PKA and CaMKII (Online Figure IV).

EPAC Is a Cardioprotective Feature of β-Adrenergic Agonsists

EPAC1 and EPAC2 are highly expressed in the heart but their functions are not well-known. They are the guanodine exchange factor for the small GTPases, Rap1 and Rap2. EPAC is thought to exert both pro-apoptotic and anti-apoptotic effects on cells depending on the cell types and conditions. The role of EPACs in cardiac myocyte apoptosis has not been reported to date. As discussed, EPACs have been suggested to be involved in myocyte apoptosis by activating the proapoptotic multifunctional CaMKII in cardiac myocytes exposed to β-adrenergic agonists. Our studies clearly indicate that prolonged treatment of cultured feline myocytes with 0.1 μmol/L 8-cp-TOME (a concentration sufficient to activate EPAC but not PKA; Figure 7A) does not induce myocyte death (Figure 7B). Our results show that EPAC protects myocytes from death induced by high extracellular Ca²⁺. EPAC-mediated ERK1/2 activation is linked to this protective effects. ERK1/2 activation downstream to EPAC activation has been reported in other cell types.

PKI inhibition of PKA Protects the Heart After MI

Excessive adrenergic activation in cardiac disease is linked to heart failure progression, and β-AR antagonists improve outcome in CHF patients. Part of the beneficial effect offered by PKA inhibition may be related to both the reduction of Ca²⁺ influx and the inhibition of SR Ca²⁺ uptake to prevent SR Ca²⁺ overload. In contrast, the effectiveness of LTCC blockers in treating failing hearts is controversial. This discrepancy could be because of the fact that LTCC blockers exert more potent effects on the vascular system than on the heart at the clinical doses. LTCC blockers also have negative inotropic effects that have to be avoided when treating the diseased heart. These effects cause a reactive excitation of the sympathoadrenergic system to increase serum catecholamines and even cardiac attack. PKA inhibition effectively and specifically reduces Ca²⁺ influxes in cardiac myocytes. Therefore, clinically used doses of LTCC blockers cannot be as effective as cardiac-specific PKA inhibition to reduce Ca²⁺ influx and SR Ca²⁺ content to protect stressed hearts.

PKA inhibition also could protect post-MI hearts by decreasing the heart rate to reduce energy consumption. Although at baseline we did not observe differences in heart rates between control and DTG mice (basal heart rate: control 560±16 bpm, n=8; DTG: 544±15 bpm, n=8), DTG hearts had reduced response to ISO stimulation (heart rate after ISO 2 mg/kg body weight, intraperitoneal: control 752±24 bpm, n=8; DTG: 603±14 bpm, n=8). Our study suggests that the detrimental aspects of β-AR signaling in HF are mediated by PKA signaling, whereas parallel PKA-independent signaling is cardioprotective. Our data indicate that beneficial effects of β-adrenergic activation would be reduced with the use of β-adrenergic antagonists (Figure 8). These beneficial effects...
could result from cAMP-mediated effects via EPAC or from biased ligand effects shown by others.39

Conclusion
The present study shows that chronic exposure of the heart to β-adrenergic agonists, as occurs in heart failure, causes myocyte death. The mechanism involves CAMP-mediated activation of PKA and resultant increase in Ca2+ influx and SR Ca2+ load. Ca2+-mediated activation of CaMKII is dependent on PKA activation. β-adrenergic-mediated increase in cAMP also activates EPAC to induce a cardioprotective effect. These results suggest that selective inhibition of excessive PKA activation could be an effective CHF therapy.

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Disclosures
None.

References


**What Is Known?**
- The β-adrenergic/sympathetic system is constantly activated when the heart is under stress.
- Persistently activated β-adrenergic system induces chronic loss of contractile heart cells (cardiomyocytes) via apoptosis.
- Protein kinase A (PKA), Ca2+/calmodulin-dependent kinase II (CaMKII), and exchange protein directly activated by cAMP (EPAC) are activated during chronic β-adrenergic stimulation and CaMKII is required for β-adrenergic agonist–induced cardiac myocyte apoptosis, but the roles of PKA and EPAC are still controversial.

**What New Information Does This Article Contribute?**
- PKA inhibition prevents myocyte death induced by β-adrenergic stimulation and myocardial infarction in vivo and in vitro.
- CaMKII is activated by increased cytosolic Ca2+ that is brought about by PKA to mediate myocyte apoptosis.
- EPAC activation in the presence of PKA inhibition protects cardiomyocytes from death through the prosurvival ERK signaling pathway.

Chronic activation of the β-adrenergic system after cardiac stress leads to the loss of contractile heart cells (cardiomyocytes), an important contributor to the progression of heart disease. Although PKA, CaMKII, and EPAC are 3 effectors activated by the β-adrenergic system, the roles of these molecules in β-adrenergic-induced myocyte death are controversial. In the study, we used a genetic tool to suppress PKA activity in cardiomyocyte in a transgenic mouse model and in cultured myocytes to explore their roles. We have proven that PKA is an important mediator of myocyte death and myocardial remodeling after β-adrenergic agonist challenge and myocardial infarction. The proapoptotic mediator CaMKII during β-adrenergic stimulation is activated by increased cellular Ca2+ caused by PKA. Furthermore, for the first time, we show that EPAC activation protects myocytes from death through the prosurvival ERK signaling and that PKA inhibition provides better protection than metoprolol at the dose offering similar PKA activity reduction. Collectively, our data show that the β-adrenergic system carries both detrimental and protective effects when activated, which is a novel finding. Therefore, PKA inhibition as a strategy to abolish the harmful signaling but to preserve the protective signaling could be a novel approach for heart disease treatment.
Cardiotoxic and Cardioprotective Features of Chronic $\beta$-Adrenergic Signaling
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SUPPLEMENTAL DATA

Materials and Methods

**PKI-GFP fusion gene and adenovirus (AdPKI) production:** A DNA oligo corresponding to the coding sequence for amino acids 1-25 (MTDVETYADFIASGRTGRRNAIHD) of mouse protein kinase (cAMP-dependent, catalytic) inhibitor α (PKIα) (mouse Entrez gene ID 18767) was synthesized and subcloned into plasmid pAcGFP1-N1 (Clontech) to make a PKI-GFP fusion gene. Amino acids 1-25 of PKIα have the PKA inhibitory domain but not the nuclear export signal. Then an adenovirus containing the fusion gene was made and further amplified in HEK293 cells. AdPKI-GFP was purified with Adeno-X purification kit and titrated with Adeno-X Rapid Titration kit (Clontech). We have successfully produced this adenovirus with a titer of 10^{10}-10^{12} pfu/ml. An adenovirus carrying GFP gene alone (AdGFP) was amplified, titrated the same way and used as control.

**Transgenic mouse model overexpressing PKI-GFP and isoproterenol treatment:** Transgenic mice overexpressing PKI-GFP was made with the inducible, cardiac specific transgenic mouse system developed by the Robbin’s group. In this system, the transgene can be expressed only in mice containing both the driver gene tTA and the responder gene PKI-GFP (double transgenic, DTG) without doxycycline. Littermate mice without any transgene (wild type, WT) or with only one transgene (tTA or PKI-GFP; single transgenic, STG) with matched age and sex were used as controls. To prevent the effects of PKA inhibition on cardiac development, PKI-GFP transgene expression was turned off during fetal and postnatal developmental stages by supplying doxycycline (625ppm) containing chow to the breeding parents and pre-weaning pups. In this system, doxycycline is gradually cleared out of the animal after weaning and transgene expression usually begins at around the age of 2 months and reaches maximal expression levels at the age of 4 months. Mice at the age of 4 months when PKI-GFP is fully expressed were used. To study short-term ISO effect on myocyte death, a single dose of isoproterenol (ISO, 60 mg/kg body weight (BW), i.p.) was injected and the heart was harvested and fixed at 8 hours post injection. To examine long-term ISO effect on myocyte death, ISO (60 mg/kg/day) was delivered by osmotic minipump (Alzet model #1002) for 3 weeks and then the heart was harvested and fixed. Cardiac function and morphology before and 3 weeks after ISO infusion were evaluated with echocardiography with a Vevo 707 machine (Visusonics, Toronto, Canada). Ejection fraction was measured with M-mode to indicate the contractility of the heart. Fixed hearts were sliced into 1mm-thick slices and processed, embedded and sectioned according to standard histological procedure.

**Myocardial infarction and treatments:** Myocardial infarction was introduced by ligating the left anterior descending coronary artery at the middle level in 4 month old PKI DTG and control mice as described previously. Echocardiography was performed 2 days before the surgery and then every week after surgery. MI mice were divided into four groups for different treats: control and PKI DTG mice receiving saline or metoprolol (20 mg/kg BW/day) daily injection for 4 weeks after MI. The dose of metoprolol that inhibits PKA activity to the same level as our PKI was used.

**Histology:** Hearts were harvested and fixed at specified time points with perfusion fixation as described previously. Cardiac tissue was processed, embedded and sectioned according to standard histological procedure. Masson’s Trichrome staining and TUNEL staining on sections were performed as previously described. The extent of fibrosis was evaluated by a person blind to mouse genotype information with a 0-10 scale.
**Adult Feline Ventricular Myocyte isolation:** Adult feline left ventricular myocytes (AFVMs) were isolated as described previously\(^9\) and the percentage of Ca\(^{2+}\)-tolerant rod-shaped myocytes were greater than 70%.

**Cell culture, transfection with adenovirus and cell counting:** Isolated AFVMs were washed 3 times with a serum-free medium (Medium 199, Sigma, St. Louis, MO) supplemented with penicillin (100,000 U/L, Sigma), streptomycin (100 mg/L, Sigma), and gentamicin (50 mg/L, Sigma) and diluted to 40,000 myocytes/ml. AFVMs were then pre-plated for 1-2 hours to eliminate fibroblasts. AFVMs were plated onto laminin-coated cover slips or culture plates (10\(^5\) VMs/well in 6-well culture plates, or 4x10\(^5\) VMs/well in 4-well culture plates). AdGFP or AdPKI was added to each well at the MOI as desired. The percentage of rod-shaped VMs was considered as the survival rate of myocytes\(^9\). Once PKI-GFP or GFP is fully expressed, VMs were treated with drugs. The numbers of rod-shaped VMs, hypercontracted VMs and spontaneously contracting rod-shaped VMs were counted in 5 randomly selected fields from each well at 24, 48, 72 and 96 hours post drug treatment. The expression of GFP or PKI-GFP was monitored with standard fluorescent microscopy and Western blotting. To increase Ca\(^{2+}\) concentration in medium M199 (1.36mM Ca\(^{2+}\)) to 2.5mM for inducing apoptosis, 2.28ml sterile 0.5M CaCl\(_2\) was added to 1L M199.

**Measurement of PKA activity and cellular cAMP production:** PKA activity in AFVMs infected with AdGFP or AdPKI, and ventricles from control mice and PKI DTG was assessed with a PKA kinase activity assay kit (Assay Design, Ann Arbor, MI), following provided protocol. Cultured AFVMs or mouse ventricle samples were homogenized in lysis buffer supplemented with 0.4 mM IBMX (3-isobutyll-methylxanthine, a non-specific inhibitor of phosphodiesterases). Basal PKA activity or maximal PKA activity activated with 1\(\mu\)M cAMP in the homogenate was determined. PKA activity after 8-cp-TOME AM application was also determined in live neonatal rat ventricular myocytes infected with AdAKAR, a PKA activity FRET probe, as described previously\(^10\). To measure cAMP production induced by isoproterenol (ISO, a \(\beta\)-adrenergic agonist) in GFP-AFVMs and PKI-AFVMs, cells were cultured in 6-well plates and labeled with 2\(\mu\)l/ml [\(^3\)H]-adenine overnight at 37°C with 5% CO\(_2\). The radio-labeling medium was replaced with fresh growth medium containing 0.4mM IBMX and incubated for 10 min at 37°C. Cells were stimulated with 10 \(\mu\)M ISO for 10 min at 37°C. The reaction was terminated by replacing reaction medium with 1ml of cold stop solution (5% trichloroacetic acid, 1mM ATP and 1mM cAMP). cAMP levels were determined with a scintillator.

**TUNEL assay, DAPI staining and Fluorochrome Inhibitor of Caspases (FLICA)staining:** TUNEL (terminal uridine nucleotide end labeling) assay was performed on VMs attached to coverslips in culture or on cardiac tissue sections using DeadEnd Fluorometric TUNEL System (Promega). Nuclear DNA was stained with DAPI (Molecular Probes, Eugene, OR), and plasma membrane was stained with TRITC-lectin (Sigma). Samples were analyzed with a fluorescence microscope (Olympus). The number of TUNEL positive nuclei as a percentage of the total nuclei was counted in 10 random fields (200X), each of which had about 250 cells. To detect caspase activation in early apoptotic myocytes, FLICA (Fluorochrome Inhibitor of Caspases) staining was done with sulforhodamine FLICA apoptosis detection kit from Immunochemistry Technologies (Bloomington, Minnesota) according to the instruction.

**Myocyte contraction, Ca\(^{2+}\) transient, Sarcoplasmic Reticulum (SR) Ca\(^{2+}\) content, Ca\(^{2+}\) currents:** Simultaneous myocyte contraction and Ca\(^{2+}\) transient (indo-1 AM) measurements were done as described previously\(^9\). Indo-1 was used because its excitation and emission spectra are not significantly influenced by those of GFP (GFP control or PKI-GFP). SR Ca\(^{2+}\) content was measured with caffeine (10mM) spritzs and the peaks of the caffeine induced Ca\(^{2+}\) transients were
used as the estimate of the SR Ca\(^{2+}\) content. To determine SR Ca\(^{2+}\) content after ISO, myocytes were paced till steady state and then exposed to 1\(\mu\)M ISO. Once a stable effect of ISO was observed, a caffeine spritz was applied to measure the SR Ca\(^{2+}\) content.

Whole cell Ca\(^{2+}\) current (\(I_{\text{Ca-L}}\)) was measured in Na\(^{+}\)- and K\(^{+}\)-free solutions at 37\(^{\circ}\)C using techniques as described.

**Western blot:** Whole cell lysates were used to determine the abundance of PKI-GFP with anti-GFP antibody (Santa Cruz), total phospholamban (PLBt), PLB phosphorylated at Serine 16 (pS16-PLB) and Threonine 17 (pT17-PLB), with standard Western blotting protocol. 37 kDa GAPDH or 100 kDa sarcomeric \(\alpha\)-actinin (when the target band size was close to 37 kDa) were used as loading controls. The following antibodies were used: PLB total (Upstate Biotechnology), pS16-PLB and pT17-PLB (Badrilla Ltd, UK), GAPDH (Sigma), sarcomeric \(\alpha\)-actinin (Sigma), CaMKII (Cell Signaling Technology), pT286 CaMKII (Cell Signaling Technology), Erk1/2 and pErk1/2 (Cell Signaling), LTCC \(\alpha\)C (Neuromab), RyR2 (Fitzgerald), calsequestrin (Sigma), NCX1 (Millipore), SERCA (Sigma). Rap1 small GTPase activation was assayed with Pierce active Rap1 pull-down and detection kit.

**Pharmacology:** To induce myocyte death by \(\beta\)-adrenergic agonists, isoproterenol (ISO, 10\(\mu\)M), ISO with a \(\beta\)1-adrenergic receptor (AR) antagonist (CGP 20712A, 6\(\mu\)M), or ISO with a \(\beta\)2-AR antagonist (ICI 118,551, 2\(\mu\)M), a \(\beta\)1-AR selective agonist (dobutamine, 10\(\mu\)M), and a \(\beta\)2-AR selective agonist (fenoterol, 10\(\mu\)M) were used to challenge cultured feline myocytes. Forskolin (10\(\mu\)M) was used to activate adenylyl cyclase directly and cAMP (1\(\mu\)M) was used to activate PKA in myocyte extract. Nifedipine (13\(\mu\)M, Sigma) was used to block Ca\(^{2+}\) influx through LTCC. Inhibitors of CaMKII (KN-93, 10\(\mu\)M), SERCA2a (Thapsigargin, 10nM, Sigma), and intracellular Ca\(^{2+}\) chelator (BAPTA-AM, 1\(\mu\)M) were also used. 8-cp-TOME-AM was used to activate EPAC. An LTCC agonist, FPL 64176, was used to increase Ca\(^{2+}\) influx into AFVMs. The concentration of each drug was determined in preliminary experiments.

**Statistics:** Data in the text and tables are reported as mean±SEM. When appropriate, paired and unpaired T-test, ANOVA or ANOVA for repeated measures were used to detect significance with SAS 9.0 (SAS Institute Inc., Cary, NC). A p value of ≤0.05 was considered significant.
References:


Supplemental Figure 1. PKI transgenic mouse model. PKI single transgenic (PKI+/-) mice were crossbred with tTA single transgenic (tTA+-) mice. Genotypes of pups were determined with the primer sets as shown. Four genotypes, WT, PKI STG, tTA STG, and PKI+-tTA+- DTG were produced by this breeding scheme. DTG mice not fed with doxycycline after weaning were used experimental group and littermate WT and STG mice were used as control mice.
Supplemental Figure 2. Calcium handling protein expression in AdGFP and AdPKI-GFP infected AFVMs treated with vehicle or ISO for 24 hours. Western blots (A) of α1c, RyR2, calsequestrin, NCX1 and SERCA and quantitation of the expression of these proteins (B) in AdGFP and AdPKI-GFP infected AFVMs treated with vehicle or ISO for 24 hours. C. Chargemovement of the L-type Ca2+ channel upon depolarization to different membrane potentials from the holding potential of -50mV in AFVMs infected with AdGFP or AdPKI-GFP treated with vehicle or ISO for 24 hours.
**Supplemental Figure 3.** PKI does not directly inhibit CaMKII activity and CaMKII activation downstream to PKA requires Ca$^{2+}$ influx through the L-type Ca$^{2+}$ channel. A-D, Western blots (A, C) and average data (B, D) of total PLB, pSer16-PLB and pThr17-PLB in GFP- and PKI-AFVMs paced or unpaced, or infected with AdCav$\beta$2a, or treated with FPL (an LTCC agonist). Total CaMKII and pCaMKII were quantitated with Western blots (B and D) in GFP- and PKI-VMs treated with FPL as well. Pacing or infection with AdCav$\beta$2a or treatment with FPL increased the phosphorylation of PLB at Thr17 site but not at Ser16 site in both GFP and PKI-VMs to a similar level. E and F, Western blots and average data of total PLB, pSer16PLB and pThr17-PLB in GFP- and PKI-VMs not treated, treated with ISO or ISO+nifedipine (an LTCC antagonist). Nifedipine did not inhibit the effect of ISO on PLB phosphorylation at Ser16 site in GFP-VMs but prevented PLB Thr17 phosphorylation. When PKA activity was inhibited by PKI, ISO could not increase the phosphorylation at Ser16 and Thr17 sites on PLB.
Supplemental Figure IV

Supplemental Figure 4. PKA inhibition prevents 8-cp-TOME induced PLB phosphorylation at both PKA and CaMKII sites. AFVMs infected with AdGFP or AdPKI were exposed to 8-cp-TOME for 10 min (0.1-10 μM). Total PLB, pSer16 PLB and pThr17 PLB were measured with Western blotting (A). B. PKA activity measured with PKA activity probe (AKAR) in live neonatal rat ventricular myocytes. The change of FRET signal indicates PKA activation and 8-cp-TOME AM at 1 and 10 μM activates PKA but 0.1μM 8-cp-TOME AM did not. C. Time course of FRET signal change in response to 10μM 8-cp-TOME AM.