Beneficial Effects of Adenylyl Cyclase Type 6 (AC6) Expression Persist Using a Catalytically Inactive AC6 Mutant

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Nonstandard abbreviations: AC, adenylyl cyclase; AC6, adenylyl cyclase type 6; AC6mut, adenylyl cyclase type 6 mutant; ATP, adenosine 5'- triphosphate; cAMP, 3',5'-cyclic adenosine monophosphate; PHLPP2, pleckstrin homology domain leucine-rich repeat protein phosphatase 2; PE, phenylephrine; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; RT-PCR, reverse transcription-polymerase chain reaction; Ca^{2+}, calcium; ANF, atrial natriuretic factor; BNP, B-type natriuretic peptide; CARP, cardiac ankyrin repeat protein; Cn, calcineurin; PLB, phospholamban; cTnI, cardiac troponin I; CamKII, calcium/calmodulin-dependent protein kinase II; ERK, extracellular signal-regulated kinase; LV, left ventricle; SR, sarcoplasmic reticulum.
ABSTRACT

Cardiac-directed expression of AC6 has pronounced favorable effects on cardiac function possibly not linked with cAMP production. To determine rigorously whether cAMP generation is required for the beneficial effects of increased AC6 expression, we generated a catalytically inactive AC6 mutant (AC6mut) that has markedly diminished cAMP generating capacity by replacing aspartic acid with alanine at position 426 in the C1 domain (catalytic region) of AC6. Gene transfer of AC6 or AC6mut (adenovirus-mediated) in adult rat cardiac myocytes resulted in similar expression levels and intracellular distribution, but AC6mut expression was associated with marked reduction in cAMP production. Despite marked reduction in cAMP generation, AC6mut influenced intracellular signaling events similarly to what was observed following expression of catalytically intact AC6. For example, both AC6 and AC6mut reduced phenylephrine-induced cardiac myocyte hypertrophy and apoptosis (p<0.001), expression of cardiac ankyrin repeat protein (p<0.01) and phospholamban expression (p<0.05). AC6mut expression, similarly to its catalytically intact cohort, was associated with increased Ca$^{2+}$ transients in cardiac myocytes after isoproterenol stimulation. Many of the biological effects of AC6 expression are replicated by a catalytically inactive AC6 mutant, indicating that the mechanisms for these effects do not require increased cAMP generation.
INTRODUCTION

Adenylyl cyclase (AC) is the effector molecule for β-adrenergic receptor (βAR) and other G-protein coupled receptors in cardiac myocytes and other cells. AC regulates the conversion of adenosine 5’- triphosphate (ATP) to 3’,5’-cyclic adenosine monophosphate (cAMP) and initiates a variety of intracellular signaling events that influence heart function (Post et al., 1995; Hanoune et al., 1997; Tesmer and Sprang, 1998; Hurle, 1999). Nine isoforms of mammalian AC have been identified so far, all possessing a short intracellular amino terminus, and two large cytoplasmic domains (C1 and C2) separated by two transmembrane domains (M1 and M2) each containing 6 transmembrane spans (Sunahara et al., 1996; Hanoune et al., 1997; Tesmer and Sprang, 1998; Smit and Iyengar, 1998; Hurle, 1999). The C1 and C2 domains form the catalytic core of AC and have activity with or without the two transmembrane domains (Tang and Gilman, 1995; Yan et al., 1996; Whisnant et al., 1996). The catalytic activity of AC is regulated by many factors: GTP binding proteins, ATP, Mg$^{2+}$, glycosylation and phosphorylation (Iwami et al., 1995; Dessauer and Gilman, 1996; Tesmer et al., 1997; Dessauer et al., 1997 and 2002; Wu et al., 2001; Lin et al., 2002; Chen-Goodspeed et al., 2005). However, intracellular AC appears to interact with intracellular proteins and influence signaling independently of its catalytic activity. For example, interaction of AC type 6 (AC6) with a PH-domain leucine-rich phosphatase protein 2 (PHLPP2) inhibits PHLPP2 activity, which leads to increased Akt phosphorylation and activity (Brognard et al., 2007; Gao et al., 2009).

β-adrenergic receptor (βAR) activation or pharmacological reagents that increase cAMP have deleterious effects on the heart (Gaudin et al., 1995; Communal et al., 1998; Engelhard et al., 1999 and 2001; Bisognano et al., 2000; Singh et al., 2001). However, increased expression of AC6, which increases agonist-stimulated cAMP, has beneficial effects (Gao et al., 1999 and
suggesting that AC6 expression has biological effects that may be unrelated to cAMP generation. Using pharmacological inhibitors, our previous data support this notion (Gao et al., 2004 and 2008). Because of the inherent limitations of studies using pharmacological inhibition, we generated a catalytically inactive AC6 mutant (AC6mut) molecule by substitution of alanine for aspartic acid at position 426 in the C1 domain of the catalytic core. Based on the crystal structure of the C1C2 catalytic core resolved by Gilman’s group, this amino acid is required for magnesium binding, but not critical for the overall structure of the catalytic core (Tesmer et al., 1997). This catalytically inactive mutant of AC6 enabled us to determine whether the beneficial effects of AC6 on intracellular signaling, calcium handling, and cardiac myocyte hypertrophy and apoptosis are cAMP-independent.
MATERIALS AND METHODS

Adult rat cardiac myocyte culture and gene transfer

Adult rat cardiac myocytes were isolated from 10-week-old Sprague-Dawley rats as described previously (Patel et al., 2006). Isolated cardiac myocytes were suspended in M199 medium (Invitrogen, Carlsbad, CA) supplemented with 1% bovine serum albumin (HyClone, Logan, UT), 100 IU/ml penicillin, and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA), and were plated on laminin-coated dishes. The culture medium was changed to fresh medium to remove the damaged myocytes that failed to attach after 2 hr in the plate. At this point, about 70 - 80% myocytes were viable and rod-shaped. Gene transfer was performed by infecting cells with E1-deleted adenoviruses encoding murine AC6 (Ad.AC6) or AC6 mutant (Ad.AC6mut) at 2 x 10^7 virus particles per well of a 12-well plate. An adenovirus vector lacking a transgene insert (Ad.Null; Invitrogen, Carlsbad, CA) was used as an additional control.

AC activity

Adult rat cardiac myocytes were seeded in 12-well plates at 15,000 cells/well. After washing and changing media, about 12,000 cells remain in the well. Cells were incubated with Ad.AC6mut or Ad.AC6 for 44 hr and stimulated with isoproterenol (Sigma, 10 µM, 10 min) or forskolin (Sigma, 10 µM, 10 min) or were not stimulated (basal). Cells were lysed in lysis buffer 1B (2.5% dodecyltrimethyammonium bromide, 0.05 M sodium acetate, pH 5.8, and 0.02% bovine serum albumin). The amount of cAMP in the supernatant was measured using the cAMP Biotrak Enzymeimmunoassay System (GE Healthcare, Waukesha, WI) following the instructions from the provider.
Calcineurin activity

Calcineurin activity was assayed using a “Calcineurin cellular activity assay kit” from Enzo life Sciences. Briefly, cells were lysed in lysis buffer containing protease inhibitors, passed through a 18g needle, and centrifuged at 16,000 x g at 4°C for 60 min. Supernatant was desalted by gel filtration. After determined protein concentration, same amount of protein (1.1 μg) was used in calcineurin activity assay. The results, amount of phosphate released by calcineurin, were calculated using slope and intercept obtained from standard curve.

Immunoblotting, immunoprecipitation and immunofluorescence staining were performed as described previously (Gao et al., 2004 and 2008).

Cardiac myocyte hypertrophy and apoptosis

To determine how AC6 and AC6mut influence cardiac myocyte response to a hypertrophic stimulus, isolated adult cardiac myocytes were infected with Ad.AC6, Ad.AC6mut or Ad.Null for 24 hr followed by incubation with phenylephrine (PE, Sigma, 20 µM) for 44 hr. In alternative experiments, PE was incubated with cardiac myocytes for 24 hr followed by incubation with Ad.AC6, Ad.AC6mut, or Ad.Null for 24 hr. Cardiac myocyte size was measured from imaged photos using “Metamorph” software, an integrated morphometry analysis (Molecular Devices, Sunnyvale, CA) stipulating a cell length of ≥107 µm. Dead cardiac myocytes, defined as small, dense, and rounded cells (Merkus et al., 2005) were counted, confirmed by exclusion of trypan blue (Zaugg M, 2002), and expressed as a percentage of total cells. Cardiac myocyte apoptosis was determined using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Total cells (untreated, 700-800; PE treated, 500-600) and apoptotic cells...
were counted and expressed as percentage of total cells. The expression of fetal genes was
determined using the quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

Measurements of Cytoplasmic Ca\textsuperscript{2+}

Cardiac myocytes were isolated from adult rat and plated on laminin-coated 25-mm glass
coverslips. Cells were infected with Ad.Null, Ad.AC6, or Ad.AC6mut for 48 hr and stimulated
with isoproterenol (10 μM) for 20 hr. Cytoplasmic Ca\textsuperscript{2+} was measured as described previously
(Tang et al., 2008).

Statistical analysis

Data represent mean ± SD; group differences were tested for statistical significance using
one-way ANOVA, followed by Bonferroni t-testing. The null hypothesis was rejected when \( p < 0.05 \).
RESULTS

Expression and location of AC6mut

Both transgene AC6 and AC6mut proteins were increased 20-25-fold over endogenous AC6 in uninfected and Ad.Null controls (Fig. 1A), and showed similar distribution to plasma membrane/caveolae and sarcoplasmic reticulum (Fig. 1B).

AC6 and AC6mut activity

Neither AC6 nor AC6mut expression altered basal cAMP (AC6: 3±3 fmol/µg; AC6mut: 3±2 fmol/µg; Control: 2±1fmol/µg.) AC6 gene transfer was associated with increased cAMP production when stimulated with isoproterenol (AC6: 623±223 fmol/µg; Con: 22±6 fmol/µg; p<0.001) and forskolin (AC6: 2842 ± 133 fmol/µg; Con: 636 ± 25 fmol/µg; p<0.001) (Fig. 1C). However, AC6mut expression was associated with a 59% reduction in cAMP production stimulated by isoproterenol (AC6mut: 9±8 fmol/µg; Con: 22± 6 fmol/µg; p>0.05) and with an 80% reduction in forskolin stimulated cAMP production (AC6mut: 130±5 fmol/µg; Con 636±25 fmol/µg; p<0.001; Fig. 1C). These data demonstrate that AC6mut expression markedly reduces cAMP generating capacity and may interfere with endogenous AC activity.
Cardiac myocyte death, apoptosis and hypertrophy

Cell Death. Cultured adult cardiac myocytes contain 10% dead cells (morphological evaluation plus trypan blue confirmation) 3d after plating. Gene transfer of AC6 and AC6mut did not change cell death rate (Fig. 2B), but PE increased cardiac myocyte death 4.7-fold (Con:10±2%; Con+PE: 47±2%; p<0.001). AC6 and AC6mut gene transfer reduced PE-induced myocyte death (AC6+PE: 20±1%; AC6mut: 21±3%; Con+PE: 47±2.0%; p<0.001; Fig. 2C).

Apoptosis. PE stimulation increased cardiac myocyte apoptosis (detected by TUNEL) 5.8±1.2-fold over untreated controls (both uninfected and Ad.Null infected, p<0.001). AC6 and AC6mut gene transfer were associated with a >60% reductions in PE-induced myocyte apoptosis vs Ad.Null infected cells (p<0.001; Fig. 2D). These data indicate that AC6-related attenuation of cardiac myocyte hypertrophy and apoptosis does not require cAMP generation.

Hypertrophy. PE (20 μM for 44 hr) increased cardiac myocyte area by 2.4-fold (Ad.Null+PE: 8955 ± 2940 μm²; Ad.Null (alone): 3740 ± 887 μm²; p<0.001) without changing cell length. Gene transfer of AC6 or AC6mut did not influence cardiac myocyte area (Ad.AC6: 3643 ± 962 μm²; Ad.AC6mut: 3696 ± 990 μm²; Ad.Null: 3740 ± 887 μm²). However, both Ad.AC6 and Ad.AC6mut attenuated PE-induced cardiac myocyte hypertrophy by 36% and 31%, respectively (Ad.AC6+PE: 5762 ± 1400 μm²; Ad.AC6mut+PE: 6153 ± 1655 μm²; Con+PE: 8955 ± 2940 μm²; p<0.01; Fig. 2C).

In uninfected or Ad.Null infected cells, PE increased atrial natriuretic factor (ANF) mRNA expression by 1.5-fold, (p>0.05). Increased AC6 reduced, but not significantly, ANF expression after PE treatment. AC6mut acted similarly to Ad.Null control (Fig. 3A). PE increased B-type natriuretic peptide (BNP) mRNA by 2-fold (p<0.001). Increased AC6 and AC6mut expression
did not alter PE-induced BNP expression when compared to Ad.Null-infected and uninfected cardiac myocytes (Fig. 3B).

**CARP expression**

Next, we examined the effects of AC6 and AC6mut expression on cardiac ankyrin repeat protein (CARP), a fetal gene activated during cardiac myocyte hypertrophy (Aihara et al., 2000; Maeda et al., 2002). Ad.Null, Ad.AC6 or Ad.AC6mut gene transfer did not alter CARP expression. After PE treatment, CARP mRNA was increased in uninfected cells, unchanged in Ad.Null infected cells, but markedly reduced in cells infected with Ad.AC6 and Ad.AC6mut. CARP protein expression was also reduced by increased AC6 and AC6mut expression (Fig. 3C and 3D). Clearly increased AC6 and AC6mut expression reduce PE-induced CARP expression, through unknown mechanisms.

**Calcineurin expression and activity**

Increased expression of calcineurin A has been described in the setting of cardiac myocyte hypertrophy (De Windt et al., 2000; Bousette et al., 2010). However, we found that PE neither changed calcineurin A expression no calcineurin activity in all groups (Fig.3E and 3F). These data suggest that AC6 and AC6mut induced reduction in cardiac myocyte hypertrophy seems not through suppression of calcineurin signaling pathway.

**SR Ca$^{2+}$ storage**

To determine whether AC6 or AC6mut expression was associated with alterations in SR [Ca$^{2+}$], cardiac myocytes were analyzed by real-time [Ca$^{2+}$]cyt imaging. No group differences in
caffeine-stimulated Ca\textsuperscript{2+} transients were present (Fig. 4A). However, when stimulated with isoproterenol (1 \(\mu\)M, 24 hr), caffeine-stimulated Ca\textsuperscript{2+} transients were increased 2.4-fold in Ad.AC6 infected cardiac myocytes (P<0.001; Fig. 4B) and by 1.7-fold in Ad.AC6mut infected cardiac myocytes (p<0.01; Fig. 4B). An apparent difference between AC6 and AC6mut did not reach statistical significance. These data indicate that despite impaired cAMP generation, AC6mut has the ability to increase cardiac myocyte SR Ca\textsuperscript{2+} storage.

**Expression and phosphorylation of phospholamban**

To determine possible mechanisms the increased Ca\textsuperscript{2+} handling seen following AC6 and AC6mut gene transfer, we examined the effects of AC6 and AC6mut expression on phospholamban (PLB) and troponin I (TnI) expression and phosphorylation. Gene transfer of AC6 or AC6mut reduced PLB expression by 46\% ± 18 (p<0.05) and 41\% ± 23 (p<0.05), respectively (Fig. 5A).

The extent of PLB phosphorylation at the Ser16 or Thr17 sites in adult rat cardiac myocytes is low. Gene transfer of AC6, but not AC6mut, increased basal PLB phosphorylation at Ser16 by 7.3-fold (p<0.001; Fig. 5B). After isoproterenol stimulation, phospho-Ser16 was increased, but there were no group differences (Fig. 5B). AC6 and AC6mut gene transfer did not increase basal PLB phosphorylation at the Thr17 site. After isoproterenol stimulation, AC6, but not AC6mut, increased PLB phosphorylation at the Thr17 site 2-fold (Fig. 5C).
Cardiac troponin-I expression and phosphorylation

AC6 or AC6mut expression was not associated with alterations in cTnI expression or basal phosphorylation at p23/24. After isoproterenol stimulation, phosphorylation of TnI phosphorylation was increased by expression of AC6 (1.6-fold, p<0.001) and AC6mut (1.4-fold, p<0.001, Fig. 5D).
DISCUSSION

In this study we have shown that a single amino acid substitution in the catalytic domain (C1) of AC6 resulted in marked reduction of catalytic activity. We used this catalytically inactive mutant to determine whether the benefits of AC6 gene expression are cAMP dependent. The most important findings are that both AC6 and AC6mut attenuated PE-induced cardiac myocyte hypertrophy and cell death, reduced CARP and PLB expression, and increased Ca\(^{2+}\) handling. The striking similarity in the direction and degree of these changes between a catalytically active (AC6) and a catalytically inactive mutant (AC6mut) indicates that such alterations do not require increased cAMP. We conclude that many of the beneficial biological effects of AC6 do not require increases in cAMP.

We previously have shown that increased expression of AC6 in neonatal rat cardiac myocytes alters intracellular signaling events (Gao et al., 2004, 2008, and 2010), but the precise mechanism has remained elusive. In the present study, we confirmed that most of the effects of AC6 expression seen in neonatal rat cardiac myocytes (Gao et al., 2004, 2008, 2009 and 2010) also are present in adult rat cardiac myocytes. More importantly, in the present study, exploiting a catalytically inactive AC6 enabled us to identify the effects of AC6 expression that might be cAMP independent. This strategy was productive and provided novel information.

Cyclic AMP dependent effects

It is important to point out that, as anticipated, not all of AC6’s effects were cAMP-independent. For example: 1) After AC6 gene transfer, PE stimulation was associated with increased PLB phosphorylation at the Ser16 and Thr17 sites (Fig. 3A and 3B), a cAMP-
dependent process which did not occur after AC6mut expression; 2) AC6, but not AC6mut expression, increased isoproterenol-induced phosphorylation of PLB at Thr17, indicating CamKII activation; 3) AC6 and AC6mut expression did not prevent isoproterenol-stimulated increases in PKA-mediated signaling (PLB phosphorylation at Ser16 or TnI phosphorylation at p23/24) or CamKII-mediated signaling (PLB phosphorylation at Thr17), indicating that activation of other endogenous ACs is not inhibited by AC6mut.

**Cyclic AMP-independent effects**

How do AC6 and AC6mut influence intracellular signaling independently of increases in cAMP generation? Based on our previous studies of AC6 expression in neonatal cardiac myocytes, we speculate that increased expression of AC6 and AC6mut enable interactions with intracellular proteins that previously were inaccessible. For example, co-immunoprecipitation and immunohistology indicate close associations of intracellular AC6 with a specific phosphatase (Brognard et al., 2007; Gao et al., 2009). Others have found that the N-terminus of AC6 interacts with snapin protein, linking AC6 to snapin-binding protein complexes (Wang et al., 2009), which may enable AC6 and AC6mut to function independently of cAMP. We recently discovered that increased AC6 and AC6mut expression associate with α-B-crystallin (data not shown), although the biological consequence of this interaction is presently unknown. It is also plausible that AC6 and AC6mut are interfering with normal signaling by preferentially associating with key intracellular signaling proteins such as GPCRs, Gαs, Gβγ, and scaffold proteins such as A-kinase anchoring proteins (AKAPs) thus serving a dominant negative function, which could also influence the hypertrophic response. For example, we found that AC6mut inhibited forskolin stimulated AC activity (**Fig. 1C**). Although forskolin is a direct AC
activator, its effectiveness is influenced by the presence of \( \Gamma \alpha \). In cells lacking \( \Gamma \alpha \), forskolin stimulated cAMP production was reduced (Darfler et al., 1982). Our data suggest that AC6mut acts as a dominant negative mutant by interacting with \( \Gamma \alpha \) to reduce the effects of \( \Gamma \alpha \) on endogenous AC responsiveness to forskolin. Identification of AC6-interacting proteins and the consequences of such interactions in adult cardiac myocytes is a focus of study in our laboratories.

**Inhibition of hypertrophy**

PE, an \( \alpha_1 \)-adrenergic receptor agonist, induces cardiac myocyte hypertrophy (Simpson, 1985) and cell death. Many genes are involved in PE-induced cardiac myocyte hypertrophy. For example, transcription of cardiac ankyrin repeat protein (CARP) is increased in PE-induced hypertrophy (Pinson et al., 1993). In the present study, expression of AC6 or AC6mut reduced CARP expression and attenuated hypertrophy, through unknown mechanisms. Increased expression of AC6 in vivo was not associated with altered expression of \( \beta AR \), \( \Gamma \alpha \) or \( \Gamma \alpha_2 \) (Gao et al., 1999), so the attenuation of hypertrophy observed following expression of AC6 or AC6mut in the present study, is not likely to be mediated through the \( \beta AR \)-signaling pathway. Moreover, AC6mut and AC6, which have opposite effects on cAMP production, have similar effects on the attenuation of hypertrophy.

Adult rat cardiac myocyte hypertrophy was confirmed by measuring protein synthesis using \(^3\)H-leucine incorporation. As expected, when uninfected and Ad.Null-infected cardiac myocytes were treated with phenylephrine we saw increased apoptosis and necrosis. Protein synthesis was decreased by 20% when compared to cardiac myocytes not treated with phenylephrine. In essence, the confounding elements of increases in apoptosis and cell death make protein
synthesis measurements less desirable than evaluating hypertrophy based on size of viable cells in this model. Nevertheless, in PE-treated groups, AC6 and AC6mut gene transfer decreased cell size and reduced protein synthesis by 25% and 16% respectively.

AC6 (but not AC6mut) expression increased PLB phosphorylation at Ser16 and Thr17 in response to PE stimulation (Fig. 3A and 3B). These data suggest that PKA and CamKII are not involved in attenuation of PE-induced hypertrophy. Expression of AC6 or AC6mut did not alter the expression of hypertrophy-associated genes such as ANF and BNP, did not increase calcineurin A expression, and did not influence the ERK signaling pathway (data not shown), but inhibited CARP expression (Fig. 3C and 3D), which likely contributed to the attenuation of hypertrophy.

We recently reported that AC6 deletion reduced left ventricular hypertrophy in pressure-overloaded female mice (Tang et al., 2010), which seems counter to what we found vis-à-vis cardiac myocyte hypertrophy in the present study. However, because of the influence of targeted expression and deletion of specific genes on non-targeted proteins, it is not axiomatic that increased expression of AC6 should result in directionally opposite effects vs AC6 deletion. A simple example illustrates this point. In the AC6 targeted deletion lines, we saw a marked diminution in cardiac AC5 protein levels. AC5 mRNA expression was unchanged, but the deletion of AC6 was associated with marked increases in AC5 protein degradation (Tang et al., 2008). AC5 deletion is associated with reduced LV hypertrophy in pressure overload (Yan et al., 2007) — its absence in AC6 deleted lines may explain reduced hypertrophy in pressure overload (Tang et al., 2010). Second, by increasing the expression of AC6 (or AC6mut), the likelihood of interactions with intracellular proteins (not usually accessible to endogenous levels of AC6) is increased — a condition not present in the setting of AC6 deletion. Furthermore, the responses of
cardiac myocytes in vitro to acute changes in milieu are often not replicated by cardiac myocytes in vivo that naturally reflect more chronic responses to alterations in physiological conditions. Finally, regarding the physiological correlates of the findings of the current studies vis-à-vis hypertrophic responses in vivo, we see improved LV function following pressure overload hypertrophy in mice with cardiac-directed expression of AC6 (unpublished data), although LV hypertrophy is similar to that observed in transgene negative siblings.

**Ca\(^{2+}\) handling**

AC6 expression (but not AC6mut expression) was associated with isoproterenol-induced PLB phosphorylation at Thr17. Both AC6 and AC6mut expression were associated with reduced PLB expression and increased cytoplasmic Ca\(^{2+}\) concentration in response to caffeine stimulation (Fig. 4). Although AC6mut expression did not increase isoproterenol-stimulated PLB phosphorylation at Thr17, its beneficial effects on Ca\(^{2+}\) handling likely reflect reduced PLB expression, which was also seen after AC6 expression. Previous studies have established the tight linkage between levels of AC6 and calcium handling in the heart. For example cardiac-directed expression of AC6 was associated with increased SR Ca\(^{2+}\) uptake (Tang et al., 2004). In contrast, hearts from AC6 deleted mice show substantially impaired Ca\(^{2+}\) handling (Tang et al., 2008).

**Conclusions**

Expression of a catalytically inactive mutant of AC6 exhibited many of the beneficial biological effects seen with expression of the catalytically active normal AC6. Both AC6 and AC6mut reduced PLB expression, increased Ca\(^{2+}\) handling, and reduced PE-induced cardiac
myocyte hypertrophy and cell death. These data indicate that the favorable effects associated
with cardiac AC6 expression are not solely dependent upon increases in cAMP generation.

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Authorship Contributions
Participated in research design: Gao, T. Tang, Lai, Yuan, and Hammond
Conducted experiments: Gao, R.Y. Tang, Guo, and Firth
Contributed new reagents: Miyannohara
Performed data analysis: Gao
Wrote or contributed to the writing of the manuscript: Gao and Hammond
Other: Gao and Hammond, acquired funding for the research.
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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. AC6 and AC6mut expression, localization and activity.

A. Expression of AC6 and AC6mut proteins. Adult cardiac myocytes were incubated with Ad.AC6mut, Ad.AC6 or Ad.Null (control) for 40 hr. AC6 and AC6mut proteins were detected by anti-AC5/6 antibody in immunoblotting.

B. Location of transgene proteins. Double immunofluorescence staining of AC6 and AC6mut by anti-AU1 antibody (red), anti-caveolin 3 (Cav-3) antibody (green) and anti-protein disulphide-isomerase (PDI) antibody (green). Hoechst dye was used to identify the nucleus (blue). There were no apparent group differences in cellular distribution: AC6 and AC6mut proteins were present in plasma membrane (associated with caveolin), nuclear envelope, and sarcoplasmic reticulum.

C. AC activity. Cyclic AMP was measured in uninfected (Con), Ad.AC6 and Ad.AC6mut infected cardiac myocytes before (basal) and after stimulation with isoproterenol (Iso, 10 μM, 10 min) or forskolin (Fsk, 10 μM, 10 min). As expected, AC6 increased cAMP generation in response to isoproterenol and forskolin stimulation. AC6mut was associated with reduced cAMP generation in response to isoproterenol (a 59% reduction) and forskolin (an 80% reduction). Bars in the graphs denote mean ± SD (***, p < 0.001) derived from triplicates in three independent experiments.

Fig. 2. Cardiac myocyte cell death, apoptosis and hypertrophy

A. Phenylephrine-induced cell death. Dead cardiac myocytes were counted and expressed as a percentage of total cardiac myocytes. AC6 and AC6mut reduced PE-associated cardiac
myocyte death. Bars in graphs are mean values (***, $p < 0.001$) derived from assessment of 800-1000 cells for each condition; experiments were repeated three times.

B. **Phenylephrine-induced myocyte apoptosis.** Top panel: Apoptotic cardiac myocytes were detected using TUNEL staining and expressed as fold increase over untreated myocytes. The lower panel displays TUNEL positive cells. AC6 and AC6mut reduced PE-associated cardiac myocyte apoptosis. Bars in graphs are mean values derived from assessment of 500-800 cells for each condition; experiments were repeated three times. P values are from post hoc Bonferroni $t$-testing after one-way ANOVA (**, $p < 0.01$).

C. **Phenylephrine-induced hypertrophy.** Cardiac myocytes infected with Ad.AC6 or Ad.AC6mut were incubated with phenylephrine (20 µM, 44 hr) and cardiac myocytes were imaged and cell area ($\mu$M$^2$) measured using Metamorph, an integrated morphometry analysis program. AC6 and AC6mut inhibited PE-induced cardiac myocyte hypertrophy. P values indicate comparison to control condition with PE. Bars in graphs are mean values (***, $p < 0.001$) derived from more than 50 cells per condition; experiments were repeated three times.

**Fig. 3. Phenylephrine-induced hypertrophy: phospholamban, CARP and calcineurin**

A. **ANF mRNA** was detected by q-RT-PCR. AC6, but not AC6mut reduced ANF mRNA expression after PE treatment.

B. **BNP mRNA** was detected by q-RT-PCR. BNP mRNA was increased by PE treatment in all conditions without group differences.

C. **CARP mRNA Expression.** Real-time RT-PCR was used to determine the expression of CARP, and mRNA copy number expressed as percentage over control (uninfected and
Gene transfer of AC6 and AC6mut did not affect CARP mRNA expression. However, after incubation with PE, CARP expression was reduced by AC6 and AC6mut expression when compared with the Ad.Null control \( (p<0.05) \) or with uninfected control \( (**p<0.01) \). P values are from post hoc Bonferroni \( t \)-testing after one-way ANOVA.

**CARP protein expression.** CARP protein was detected using anti-CARP antibody in immunoblotting and showed no group differences in the absence of PE. After PE treatment, AC6 and AC6mut expression reduced expression of CARP protein when compared with both uninfected and Ad.Null controls \( (**p<0.01) \).

**Calcineurin A protein expression.** Calcineurin A protein was detected using anti-calcineurin A antibody in immunoblotting. There were no differences among groups in the basal or PE-treated conditions.

**Calcineurin activity.** Calcineurin activity was determined using the calcineurin cellular activity assay kit from Enzo Life Sciences. There were no differences among groups in the basal or PE-treated conditions.

In all graphs, bars represent mean values of 3 - 4 experiments; error bars denote 1 SD.

**Fig. 4. Calcium Signaling.** Ad.AC6mut and Ad.AC6 infected adult rat cardiac myocytes, unstimulated or stimulated with isoproterenol \( (1 \mu\text{M, 24 hr}) \), were analyzed by real-time \([\text{Ca}^{2+}]_{\text{cyt}} \) imaging. Representative \([\text{Ca}^{2+}]_{\text{cyt}} \) transients in response to caffeine stimulation were recorded with Fura-2 fluorescence.
A. **Unstimulated cardiac myocytes** expressing AC6 or AC6mut showed no change in [Ca\(^{2+}\)]\(_{\text{cyt}}\) in response to caffeine (10 mmol/L). Number of cardiac myocytes assessed: control, 34; AC6, 24; AC6mut, 31.

B. **Isoproterenol-stimulated** cardiac myocytes expressing AC6 or AC6mut showed increases in the peak amplitude of Ca\(^{2+}\) transient in response to caffeine stimulation. Number of cardiac myocytes assessed: control 19; AC6, 24; AC6mut, 17 cells. (AC6 vs. Null, ***, \(p < 0.001\); AC6mut vs. Null, **, \(p < 0.01\)).

**Fig. 5. Expression and phosphorylation of PLB and cTnI proteins**

A. **Immunodetection of total PLB protein** using anti-PLB antibody in cell homogenates of adult rat cardiac myocytes. AC6 and AC6mut reduced expression of PLB.

B and C. **Immunodetection of PLB phosphorylation at Ser16 and Thr17** using their specific antibodies in cell homogenates of adult rat cardiac myocytes unstimulated or stimulated with isoproterenol (10 µM, 24 hr). AC6, but not AC6mut, increased basal PLB phosphorylation at the Ser16 site, but not the Thr17 site. After isoproterenol stimulation, PLB phosphorylation at Ser16 was increased similarly in all conditions, and phosphorylation at Thr17 was increased in all conditions, but was greater in AC6 than in AC6mut-infected cardiac myocytes.

D. **Immunodetection of troponin I phosphorylation** at the Ser23/24 sites using anti-phospho-cTnI antibody in cell homogenates of adult rat cardiac myocytes, unstimulated or stimulated with isoproterenol (10 µM, 24 hr). AC6 and AC6mut did not increase basal cTnI phosphorylation, but isoproterenol treatment was associated with increased cTnI phosphorylation of similar
degrees. Bars in all graphs show mean values from 3 or more experiments; error bars denote 1 SD (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).
<table>
<thead>
<tr>
<th></th>
<th>Ad.AC6 vs Control</th>
<th>Ad.AC6mut vs Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso-stimulated cAMP</td>
<td>↑ 29-fold</td>
<td>↓ 59%</td>
</tr>
<tr>
<td>Fsk-stimulated cAMP</td>
<td>↑ 4.5-fold</td>
<td>↓ 80%</td>
</tr>
<tr>
<td>PE-induced hypertrophy</td>
<td>↓ 36%</td>
<td>↓ 30%</td>
</tr>
<tr>
<td>PE-induced cell death</td>
<td>↓ 57%</td>
<td>↓ 55%</td>
</tr>
<tr>
<td>PE-associated CARP protein</td>
<td>↓ 59%</td>
<td>↓ 51%</td>
</tr>
<tr>
<td>SR Ca(^{2+}) handling</td>
<td>↑ 2.4-fold</td>
<td>↑ 1.7-fold</td>
</tr>
<tr>
<td>PLB protein</td>
<td>↓ 46%</td>
<td>↓ 41%</td>
</tr>
<tr>
<td>Basal, P-Ser16-PLB</td>
<td>↑ 7-fold</td>
<td>0</td>
</tr>
<tr>
<td>Iso-stimulated, P-Thr17-PLB</td>
<td>↑ 2-fold</td>
<td>0</td>
</tr>
<tr>
<td>Iso-stimulated, P-23/24-TnI</td>
<td>↑ 1.6-fold</td>
<td>↑ 1.4-fold</td>
</tr>
</tbody>
</table>

The entries reflect summary of data from triplicate experiments in each case. “0” indicates no change; PE, phenylephrine; Fsk, forskolin; Iso, isoproterenol; PLB, phospholamban
Figure 2

A. % of cells that died

B. Apoptotic myocytes Fold/Control

C. CM Area (μM²)

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Figure 3

A

B

C

D

E

F

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Figure 4
Figure 5

A

Total PLB

% over control

Con  AC6  Mut

Total PLB

Basal

Con AC6 Mut Con AC6 Mut

0  50  100

B

P-Ser16-PLB

GAPDH

Basal Isoproterenol

Con AC6 Mut Con AC6 Mut

0  2  4  6  8

P-16-PLB/GAPDH

***

P-Thr17-PLB

GAPDH

Basal Isoproterenol

Con AC6 Mut Con AC6 Mut

0  10  20

P-23/24-TnI

Total-TnI

Basal Isoproterenol

Con AC6 Mut Con AC6 Mut

ns *** ***

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