Adenylyl Cyclase Type 6 Deletion Decreases Left Ventricular Function via Impaired Calcium Handling

Tong Tang, PhD; Mei Hua Gao, PhD; N. Chin Lai, PhD; Amy L. Firth, PhD; Toshiyuki Takahashi, MD, PhD; Tracy Guo, BS; Jason X.-J. Yuan, MD, PhD; David M. Roth, PhD, MD; H. Kirk Hammond, MD

Background—Adenylyl cyclases (ACs) are a family of effector molecules for G-protein–coupled receptors. The 2 ACs most abundantly expressed in cardiac myocytes are types 5 (AC5) and 6 (AC6), which have 65% amino acid homology. It has been speculated that coexpression of 2 AC types in cardiac myocytes represents redundancy, but the specific role of AC6 in cardiac physiology and its differences from AC5 remain to be defined.

Methods and Results—We generated transgenic mice with targeted deletion of AC6. Deletion of AC6 was associated with reduced left ventricular contractile function (\( P=0.026 \)) and relaxation (\( P=0.041 \)). The absence of AC6 was associated with a 48% decay in \( \beta \)-adrenergic receptor–stimulated cAMP production in cardiac myocytes (\( P=0.003 \)) and reduced protein kinase A activity (\( P=0.015 \)). In addition, phospholamban phosphorylation was reduced (\( P=0.015 \)), sarcoplasmic reticulum \( Ca^{2+} \)-ATPase activity was impaired (\( P<0.0001 \)), and cardiac myocytes showed marked abnormalities in calcium transient formation (\( P=0.001 \)).

Conclusions—The combination of impaired cardiac cAMP generation and calcium handling that result from AC6 deletion underlies abnormalities in left ventricular function. The biochemical and physiological consequences of AC6 deletion reveal it to be an important effector molecule in the adult heart, serving unique biological functions not replicated by AC5. (Circulation. 2008;117:61-69.)

Key Words: calcium ■ knockout mice ■ heart contractility ■ receptors, adrenergic, beta

General agreement exists that the 2 adenylyl cyclase (AC) types most abundantly expressed in cardiac myocytes are types 5 (AC5) and 6 (AC6).

When AC5 is expressed in cardiomyopathy (cardiac-directed \( Goq \) expression), \( \beta \)AR-stimulated AC activity and cardiac function are increased, but hearts retain pathological hypertrophy and develop fibrosis. In contrast, cardiac-directed expression of AC6 in this same model increases \( \beta \)AR-stimulated AC activity and cardiac function, abrogates pathological hypertrophy (no fibrosis is observed), and prolongs life. These studies suggest that AC5 and AC6 fulfill unique roles in cardiovascular function. However, a caveat should be exercised in deducing too much from studies that involve cardiac-directed transgene expression and cross-breeding strategies; differences between lines may simply reflect variations in expression levels or complex interactions of transgenes that have little to do with direct functional differences in transgenes per se. Targeted gene deletion, an alternative approach, often yields compelling data on the function of specific proteins in physiological contexts. For example, on the basis of data recently obtained from transgenic mice with AC5 deletion, it appears that AC5 may have reduced in pacing-induced congestive heart failure in dogs.1

Received July 27, 2007; accepted October 26, 2007.


The online-only Data Supplement, consisting of an expanded Methods section and figures, is available with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.107.730069/DC1.

Correspondence to H. Kirk Hammond, MD, VA San Diego Healthcare System (111A), 3350 La Jolla Village Dr, San Diego, CA 92161. E-mail khammond@ucsd.edu

© 2008 American Heart Association, Inc.

Circulation is available at http://circ.ahajournals.org DOI: 10.1161/CIRCULATIONAHA.107.730069

61
adverse effects on cardiac function,9–12 effects that previously, using other approaches (eg, gain of function), were not recognized. The possible initiation of clinical trials of AC6 gene transfer for heart failure13,14 mandates studies of AC6 role in cardiac function, not only to gain basic insight into the role of AC6 in cardiac function but also to seek possible adverse effects that may only be revealed by targeted gene-deletion studies.

In the present study, we sought to examine the biological role of AC6 in cardiac function through analysis of the consequences of targeted deletion of AC6. In addition, by comparing studies of mice absent AC6 versus AC5, we hoped to define the distinct roles of these 2 dominant AC isoforms in cardiac function.

Methods

Generation of AC6 Gene-Targeted Mice

A bacterial artificial chromosome clone containing the AC6 gene was isolated from a murine 129/SvJ genomic library by hybridization of plaque lifts with a radio-labeled mouse AC6 cDNA probe. Three overlapping AC6 gene fragments were subcloned into pBluescript II vectors for restriction mapping and sequence analysis.

The targeting vector carries a PGKneo-bpA selection cassette flanked by a 2.0-kb upstream sequence and a 3.3-kb downstream sequence of the AC6 exon 1 (Figure 1A). R1 embryonic stem cells electroporated with SacII-linearized targeting vector were selected by heterozygous pairs. Estimated sizes of PCR products are 0.6 kb for the wild-type allele with use of P3/P4 primer pairs and 2.2 kb for mutated allele with use of P1/P2 primer pairs. C, Representative Southern blot of StuI-digested tail genomic DNA from littermates generated by heterozygous pairs. Estimated detectable band sizes with a 0.4-kb probe are 5.3 and 4.1 kb, respectively, for wild-type and mutated alleles. WT indicates wild type.

Reverse-Transcription PCR

Total RNA extraction and reverse-transcription (RT) reaction were performed as described previously.11 The primer pairs used for PCR analysis were designed to cross intron-exon boundaries to eliminate potential contamination of genomic DNA. Details of PCR condition and primer sequences are listed in the online-only Data Supplement.

Echocardiography

Echocardiography was performed as described previously.15 Data were acquired and analyzed without knowledge of group identity.

Left Ventricular Contractile Function

Left ventricular (LV) contractile function and relaxation were measured in isolated perfused hearts by methods described previously.7 Data were acquired and analyzed without knowledge of group identity.

Calcium Uptake

Initial rate of ATP-dependent sarcoplasmic reticulum (SR) Ca2+ uptake was measured by the modified Millipore filtration technique as described previously.16

Measurements of Cytoplasmic Ca2+

Cardiac myocytes were isolated from adult mice as described previously11 and plated on laminin-coated 25-mm glass coverslips. Cells were loaded with the Ca2+-sensitive fluorescent indicator Fura-2 AM (3 μmol/L) at 37°C for 30 minutes, with 5% CO2 in the dark. Cells then were placed in a perfusion chamber on the stage of an inverted microscope (Nikon, Melville, NY) and superfused with Tyrode’s solution (10 mmol/L HEPES, pH 7.3, 137 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L CaCl2, 1 mmol/L MgCl2, 10 mmol/L glucose) for 20 minutes to remove extracellular dye and allow intracellular esterases to cleave cytosolic Fura-2 AM to active Fura-2. Fura-2 fluorescence was detected as 510-nm-wavelength light emission with excitation wavelengths of 340 and 380 nm by use of the digital fluorescence imaging system from Intracellular Imag-
ing (Cincinnati, Ohio). Fluorescence from 2 to 3 cytoplasmic regions in each cell was monitored continuously and averaged. Cytoplasmic Ca$^{2+}$ ([(Ca$^{2+}$)-jcyt]) was expressed (background subtracted) as Fura-2 fluorescence emission ratio excited at 340 and 380 nm (F/F$_0$). To assess SR calcium load, caffeine-induced calcium release was initiated by addition of 10 mmol/L caffeine to Tyrode’s solution. Peak Ca$^{2+}$ transients were calculated from the baseline, averaged 10 to 20 ms before the transient rise. Data were acquired and analyzed without knowledge of group identity.

**cAMP Generation**

cAMP production in LV homogenates and in isolated cardiac myocytes was measured as described previously.$^{11,17}$ Additional experiments to measure cAMP production were also performed with myocardial homogenates from neonatal mice (1 day old). Inhibition of AC activity by Ca$^{2+}$ was performed at different free Ca$^{2+}$ concentrations maintained by 0.5 mmol/L EGTA buffer.

**Protein Kinase, Phosphatase Activities, and Western Blotting**

Activities of intrinsic protein kinase A (PKA) and protein phosphatases 1 (PP1) and 2A (PP2A) were measured as described previously.$^{11,16}$ Additional experiments were conducted with LV samples from isolated hearts perfused with dobutamine (10 $\mu$mol/L, 5 minutes).

**Caspase 3/7 Activity**

LV samples were homogenized at 4°C in buffer A (25 mmol/L Tris-HCl, pH 7.4, 0.5 mmol/L EDTA, 0.5 mmol/L EGTA, 10 mmol/L $\beta$-mercaptoethanol, 50 mmol/L $\beta$-glycerophosphate, 1 mmol/L Na$_2$VO$_4$) in the presence of protease inhibitor cocktail (Roche, Indianapolis, Ind). LV homogenates were cleared by centrifugation at 13 000 rpm for 15 minutes at 4°C, and caspase 3/7 activity was measured with a Caspase-Glo 3/7 Assay (Promega, Madison, Wis).

**Blockage of AC5 Protein Proteasomal Degradation**

Isolated cardiac myocytes (75% to 90% viability) were plated on laminin-coated dishes in M199 media supplemented with 4% fetal bovine serum. After incubation (37°C, 1 hour, 5% CO$_2$), attached cells were washed, and culture medium was changed (M199 media with 1% BSA). Western blotting was performed after treatment with 10 $\mu$mol/L proteasomal inhibitor MG132 (N-carbobenzyloxy-L-leucinyl-L-leucinyl-L-leucinal; Sigma, St. Louis, Mo) or lysosomal inhibitor E64 (trans-epeoxysuccinyl-L-leucylamido(4-guanidino)butane; Sigma) for 16 hours.

**Apoptosis and Fibrosis**

A short-axis midwall LV ring (1 mm thick) was fixed in formalin for 24 hours, paraffin embedded, sectioned (6 $\mu$m), and mounted on glass slides. The terminal dUTP nick end-labeling (TUNEL) assay was performed as described previously.$^{18}$ To assess inflammation and fibrosis, sections were stained with Masson’s trichrome. A short-axis midwall LV ring (1 mm thick) was fixed in formalin for 24 hours, paraffin embedded, sectioned (6 $\mu$m), and stained with a short-act actin antibody (Sigma, St. Louis, Mo). The terminal dUTP nick end-labeling (TUNEL) assay was performed after treatment with 10 $\mu$mol/L proteasomal inhibitor MG132 (N-carbobenzyloxy-L-leucinyl-L-leucinyl-L-leucinal; Sigma, St. Louis, Mo) or lysosomal inhibitor E64 (trans-epeoxysuccinyl-L-leucylamido(4-guanidino)butane; Sigma) for 16 hours.

**Statistical Analysis**

Results were recorded as mean±SEM. Data obtained from isolated perfused hearts were analyzed with 2-way repeated-measures ANOVA, and data obtained from calcium uptake assays were analyzed with 2-way ANOVA. All other group comparisons were made with the Student t test (unpaired, 2-tailed). The null hypothesis was rejected when $P<0.05$.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Generation of Mice**

The murine AC6 gene is encoded by 21 exons within 18 kb of genomic DNA. We constructed a targeting vector, with exon 1 replaced by the PGKneo-bpA cassette, to disrupt the AC6 gene by homologous recombination (Figure 1A). Interbreeding of the heterozygote mice resulted in offspring with all 3 genotypes (Figure 1B and 1C) in Mendelian proportions (64 AC6$^{+/+}$, 129 AC6$^{+/−}$, and 68 AC6$^{−/−}$), which indicates that AC6 protein is not required for embryonic development. AC6$^{+/−}$ mice were fertile, and no physical abnormalities were obvious. Up to 10 months of age (duration of study), no mortality occurred in either group. No group differences were present in body weight, tibial length, heart weight, or lung weight at 6 to 10 months of age (Table 1). Normal heart weight/body weight ratio and heart weight/tibial length ratio indicated no cardiac hypertrophy was associated with AC6 deletion at this age.

**Cardiac AC Isoform Expression**

We performed RT-PCR and confirmed that AC6 mRNA expression was absent in LV samples from AC6$^{−/−}$ mouse hearts (Figure 2A). We then determined, using quantitative real-time RT-PCR, whether compensatory changes had occurred in the cardiac expression of other AC isoforms$^9$ (ie, AC2, AC3, AC4, AC5, AC7, and AC9) after AC6 deletion. The absence of suitable AC type-specific antibodies precluded quantitative assessment of protein content. We found that AC6 deletion did not alter mRNA expression of any of these cardiac isoforms (Figure 2B). We also performed Western blotting to confirm the absence of AC6 protein in AC6$^{+/−}$ mouse hearts. We used an antibody that recognized the c-terminus of both AC5 and AC6 proteins, because AC6-specific antibodies are not commercially available. These studies confirmed the absence of AC6 protein but also indicated marked diminution in AC5 protein, even though AC5 mRNA levels were unchanged by AC6 deletion (Figure 2C).

**Table 1. Morphometric Measurements**

<table>
<thead>
<tr>
<th></th>
<th>Control (n=16)</th>
<th>AC6$^{−/−}$ (n=16)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>34.8±2.0</td>
<td>30.3±1.1</td>
<td>0.06</td>
</tr>
<tr>
<td>Heart weight (LV+IVS), mg</td>
<td>119.2±7.8</td>
<td>108.1±7.0</td>
<td>0.30</td>
</tr>
<tr>
<td>Lung weight, mg</td>
<td>155±6</td>
<td>155±6</td>
<td>1.00</td>
</tr>
<tr>
<td>Tibial length, mm</td>
<td>18.4±0.2</td>
<td>18.1±0.2</td>
<td>0.30</td>
</tr>
<tr>
<td>HW/BW, mg/g</td>
<td>3.44±0.15</td>
<td>3.55±0.16</td>
<td>0.62</td>
</tr>
<tr>
<td>HW/tibial length, mg/mm</td>
<td>6.47±0.41</td>
<td>5.99±0.40</td>
<td>0.41</td>
</tr>
<tr>
<td>Lung weight/BW, mg/g</td>
<td>4.56±0.29</td>
<td>5.15±0.21</td>
<td>0.11</td>
</tr>
</tbody>
</table>

IVS indicates interventricular septum; BW, body weight; and HW, heart weight (LV plus IVS).

Probability values are from Student t test (unpaired, 2-tailed). Values represent mean±SEM.

**AC5 Protein Turnover**

To gain insight into the mechanism by which AC6 deletion decreased AC5 protein content, we asked whether AC6 deletion was associated with increased proteasomal or lysosomal protein turnover. We treated isolated cardiac myocytes from adult AC6$^{−/−}$ mice with MG132 (a specific proteasomal inhibitor) and E64 (a specific lysosomal inhibitor) and found that MG132
AC6 deletion increases AC5 protein turnover through facilitation of proteasomal degradation. This alteration in protein degradation was not affected by βAR stimulation with dobutamine and appeared to be limited to AC5, because other LV proteins that we assessed in the present study (PKA, phospholamban [PLN], PP1, PP2A, Gas, Gai, sarcoplasmic reticulum Ca2+-ATPase [SERCA2a], calsequestrin, RyR2, Akt, and GAPDH) showed no group differences.

Reduced cAMP Generation
Absence of AC6 protein did not affect basal cAMP levels in LV samples, but a 58% reduction occurred in cAMP production stimulated by isoproterenol and GTP·S, a nonhydrolyzable GTP analog (Figure 3A). Forskolin and forskolin derivative NKH477–stimulated cAMP production were reduced by 60% and 69%, respectively (Figure 3A). Reduced AC activity was also found in myocardium from AC6-deleted neonatal mice (Figure 1, online-only Data Supplement). AC6 deletion impaired Ca2+-mediated inhibition of AC activity (Figure 3B). Western blotting showed no change in LV protein contents of Gas (Figure 3C) or Gai (data not shown) after AC6 deletion, which indicates that AC6 deletion directly reduces AC activity.

To confirm that AC activity in cardiac myocytes was reduced after AC6 deletion, we compared cAMP generation in isolated cardiac myocytes from adult AC6+/− mice and their age- and sex-matched control littermates. Cardiac myocytes with AC6 deletion showed no reduction of basal cAMP levels, but a 70% reduction in NKH477-stimulated cAMP generation was observed (Figure 3D). We also found reduced cAMP generation in response to a wide range of dobutamine concentrations (Figure 3E). Carbachol (10 μmol/L), a muscarinic cholinergic receptor agonist, decreased isoproterenol-stimulated cAMP production.

Figure 2. Detection of AC isoforms. A, AC6 mRNA expression was undetectable in AC6+/− mouse hearts by RT-PCR. B, Quantitative real-time RT-PCR analysis of mRNA expression of cardiac AC isoforms. Contents of AC2, AC3, AC4, AC5, AC7, and AC9 were not altered by AC6 deletion (n=4). +/+ indicates control; −/−, AC6−/−. C, AC5 and AC6 protein were undetectable in AC6−/− mouse hearts by Western blotting. D, MG132 (MG), a specific inhibitor of proteasomal degradation, restored AC5 content to normal levels in cardiac myocytes from AC6−/− (Figure 2D). Treatment with E64, in contrast, did not change treatment was associated with increased AC5 protein content (Figure 2D). Treatment with E64, in contrast, did not change AC5 protein content (Figure 2D). These results indicate that

Figure 3. A, AC6 deletion did not change basal cAMP, but stimulation with isoproterenol (iso; 10 μmol/L) plus GTP·S (10 μmol/L), forskolin (10 μmol/L), and NKH477 (10 μmol/L) showed reduced cAMP production in LV samples (n=8); B, AC6 deletion reduced Ca2+-mediated inhibition of AC activity in LV homogenates. Experiments were conducted in the presence of isoproterenol (10 μmol/L) and GTP·S (10 μmol/L; n=6). AC activity at 0.32 μmol/L free Ca2+ was set as 100%. C, AC6 deletion did not affect Gas expression in LV samples. Upper panel, a representative Western blot; Lower panel, quantification of data from Western blotting (n=8). D, Cardiac myocyte cAMP production in response to NKH477 (10 μmol/L) stimulation was reduced by AC6 deletion (n=5). E, AC6 deletion was associated with impaired cAMP production in isolated cardiac myocytes through a wide range of dobutamine concentrations (n=5). F, Carbachol (C) inhibition of isoproterenol (I; 10 μmol/L)-stimulated cAMP production was impaired in cardiac myocytes isolated from AC6−/− mice (n=3). Probability values shown for B and E are from 2-way ANOVA (control vs AC6−/−). CON indicates control; ○, control; and ●, AC6−/−.
Table 2. Echocardiographic Measurements

<table>
<thead>
<tr>
<th></th>
<th>Control (n=23)</th>
<th>AC6−/− (n=22)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDD, mm</td>
<td>3.70±0.07</td>
<td>3.40±0.09</td>
<td>0.38</td>
</tr>
<tr>
<td>ESD, mm</td>
<td>2.21±0.08</td>
<td>2.19±0.08</td>
<td>0.86</td>
</tr>
<tr>
<td>PW, mm</td>
<td>0.65±0.01</td>
<td>0.65±0.01</td>
<td>1.00</td>
</tr>
<tr>
<td>IVS, mm</td>
<td>0.67±0.01</td>
<td>0.69±0.01</td>
<td>0.16</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>Basal 459±15</td>
<td>463±11</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>Dobutamine 559±10</td>
<td>553±7</td>
<td>0.63</td>
</tr>
<tr>
<td>FS, %</td>
<td>Basal 40.6±1.3</td>
<td>39.4±1.3</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>Dobutamine 65.3±1.2</td>
<td>56.6±1.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>Basal 78.3±1.4</td>
<td>77.1±1.4</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>Dobutamine 95.5±0.5</td>
<td>91.4±0.8</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

EDD indicates end-diastolic diameter; ESD, end-systolic diameter; PW, posterior wall; IVS, interventricular septum; HR, heart rate; FS, fractional shortening; and LVEF, LV ejection fraction. Probability values are from Student t test (unpaired, 2-tailed). Values represent mean±SEM.

Reduced LV Function
Echocardiography revealed that AC6 deletion did not affect basal LV fractional shortening or basal LV ejection fraction, but fractional shortening and LV ejection fraction during dobutamine stimulation were diminished in AC6-deleted mice (Table 2). Basal and dobutamine-stimulated heart rates were not changed by AC6 deletion (Table 2).

To assess the effect of AC6 deletion on intrinsic cardiac contractile function independent of autonomic reflex activation and endogenous catecholamines, we measured LV pressure development and decay in isolated perfused hearts. AC6 deletion was associated with reductions in LV developed pressure (P=0.019; Figure 4A) and LV +dP/dt during dobutamine stimulation (P=0.026; Figure 4B). Both basal LV pressure (control 109±7 mm Hg; AC6−/− 92±6 mm Hg; P=0.08; n=9) and basal LV +dP/dt (control 2961±204 mm Hg/s; AC6−/− 2327±233 mm Hg/s; P=0.06; n=9) tended to be lower in mice with AC6 deletion (Figure 4A and 4B). AC6 deletion was associated with decreased LV −dP/dt in response to dobutamine stimulation (P=0.041; Figure 4C); basal LV −dP/dt was unchanged (control −1814±119 mm Hg/s; AC6−/− −1503±154 mm Hg/s; P=0.13; n=9); and heart rates were similar (Figure 4D).

Decreased LV PKA Activity and PLN Phosphorylation
To determine whether AC6 deletion was associated with alterations in more distal aspects of βAR signaling, we evaluated PKA activity and PLN phosphorylation. PKA activity was reduced in AC6−/− mice (P<0.015; Figure 5A), although Western blotting showed no difference in protein content of the PKA catalytic subunit (Figure 5B). Reduction of PKA activity was also observed in isolated mouse hearts perfused with 10 μmol/L dobutamine for 5 minutes (control 918±104 pmol · mg−1 · min−1; AC6−/− 563±72 pmol · mg−1 · min−1; P=0.03; n=4).

We then determined whether decreased PKA activity in AC6−/− mice was associated with decreased phosphorylation of PLN, a PKA substrate. By Western blotting, we found that AC6 deletion was associated with decreased protein content of Ser16 phosphorylation of PLN (P=0.019; Figure 4A) and LV −dP/dt in response to βAR stimulation. C, AC6 deletion decreased LV −dP/dt in response to βAR stimulation. D, AC6 deletion did not alter basal and βAR-stimulated heart rate (HR). Probability values shown are from 2-way repeated-measures ANOVA (control vs AC6−/−), n=9. ○, control; ●, AC6−/−.

Abnormal LV Calcium Uptake
To determine whether AC6 deletion altered LV Ca2+ uptake, we compared ATP-dependent initial SR Ca2+ uptake rates in LV homogenates from control and AC6−/− mice at multiple Ca2+ concentrations (Figure 6A) and decreased SERCA2a affinity for Ca2+ (EC50: control 0.76±0.03 μmol/L; AC6−/− 0.15±0.03 μmol/L; P=0.019). AC6 deletion decreased SV2 AR-stimulated heart rate, 78.3±1.4% vs 77.1±1.4% (P=0.55; n=23). Basal and dobutamine-stimulated heart rates were not changed by AC6 deletion (Table 2).

Tang et al AC6 Knockout 65

Figure 4. Cardiac responsiveness to βAR stimulation. A, AC6 deletion decreased LV developed pressure in isolated perfused hearts from control and AC6−/− mice at various concentrations of dobutamine. LVP indicates LV pressure. B, AC6 deletion decreased LV +dP/dt in response to βAR stimulation. C, AC6 deletion decreased LV −dP/dt in response to βAR stimulation. D, AC6 deletion did not alter basal and βAR-stimulated heart rate (HR). Probability values shown are from 2-way repeated-measures ANOVA (control vs AC6−/−), n=9. ○, control; ●, AC6−/−.
2.68±0.08 μmol/L; P<0.0001; n=8; Figure 6B). Protein contents of SERCA2a and calsequestrin were not altered by AC6 deletion (Figure 6C and 6D).

Cardiac RyR2 Phosphorylation
To explore the possible role of AC6 in Ca²⁺ release from SR, we compared RyR2 phosphorylation and protein content in AC6-/- mice and their control littermates. Neither RyR2 phosphorylation (control 420±28 DU; AC6-/- 423±58 DU; P=0.96; n=8) nor RyR2 protein content (control 880±55 DU; AC6-/- 958±120 DU; P=0.56; n=8) was altered by AC6 deletion. In addition, no group difference was present in RyR2 phosphorylation in dobutamine-perfused hearts (control 468±195 DU; AC6-/- 438±73 DU; P=0.89; n=4).

Reduced SR Ca²⁺ Storage
To determine whether defective SR Ca²⁺ uptake that resulted from AC6 deletion could alter intracellular [Ca²⁺], isolated cardiac myocytes were analyzed by real-time [Ca²⁺]cyt imaging (Figure 7A). No difference was present between basal intracellular Ca²⁺ levels in cardiac myocytes from AC6-/- mice and their intact control littermates (Figure 7B). However, caffeine-stimulated Ca²⁺ transients were reduced by 50% in cardiac myocytes from AC6-deleted mice (P=0.001; Figure 7C). These data, together with the finding of decreased SR Ca²⁺ uptake, indicate that cardiac myocyte SR Ca²⁺ storage is markedly impaired by the deletion of AC6.

Akt Activation
LV samples from AC6-deleted mice showed reduced Akt phosphorylation (Figure 8; P<0.0001), although no group difference (control versus AC6-/-) was found in Akt phosphorylation after 5 minutes of dobutamine stimulation (Figure II, online-only Data Supplement). Akt protein content was not altered (Figure 8C). TUNEL staining was performed to determine whether AC6 deletion affected cardiac myocyte apoptosis. The incidence of apoptosis was quite low and similar in LV samples from both groups (control 13±38 apoptotic cells/10⁶ total myocytes; AC6-/- 13±38 apoptotic cells/10⁶ total myocytes; P=1.0; n=4). There also were no group differences in
caspase 3/7 activities (control 15.8±3.8 relative light units [RLU]/μg; AC6−/− 15.3±3.4 RLU/μg; P=0.18; n=8). Mason’s trichrome staining showed no evidence of inflammation or fibrosis in transmural LV samples from either group.

Discussion

Targeted deletion of AC6 is associated with abnormal LV systolic and diastolic function. In studies conducted in LV membranes and cardiac myocytes, marked impairment of βAR-stimulated cAMP generation, similar in degree to that first described in failing human hearts,19 was found. In addition, abnormalities in LV contractile responsiveness and profound decrements in multiple levels of calcium handling were observed. The physiological consequences of AC6 deletion on the heart are not only qualitatively and quantitatively different from those seen in AC5 deletion, they are often directionally opposite. For example, LV calcium handling was improved by AC5 deletion11 but decreased by AC6 deletion. These results indicate that AC6 fulfills a unique and important biological role in the adult heart.

Impaired Calcium Handling

Current understanding of the role of AC6 in intact heart comes primarily from studies that use cardiac-directed expression of AC6 in transgenic mice or AC6 gene transfer.4,5,7,8,15,17,18,20 Generation of mice with targeted AC6 deletion enabled us to see its physiological consequences and explore the mechanisms for abnormalities in LV function for the first time. AC6 disruption had profoundly negative effects on LV calcium handling, effects that reverberated from the cellular to the organ level. For example, LV samples from AC6-deleted mice showed marked reductions in PLN phosphorylation (P<0.015) and SERCA2a activity (P<0.0001), and cardiac myocytes showed striking abnormalities in caffeine-stimulated calcium transient formation (P<0.001), which indicates marked impairment of SR calcium storage capacity. Physiological abnormalities, which included diminished LV force generation and relaxation and blunted LV contractile responsiveness, occurred as a result of impaired cardiac cAMP generation and markedly disturbed calcium handling. By inference, endogenous AC6 plays an essential role in LV calcium handling and, consequently, in LV force generation and relaxation in the normal heart.

We previously reported that cardiac-directed AC6 expression increases PKA activity, PLN phosphorylation, and SERCA2a affinity for Ca2+ in failing hearts.18 In the present study, we found that AC6 disruption results in reductions in each of these elements. These data from complementary models using AC6 expression and AC6 deletion are consistent with the hypothesis that AC6 predominantly affects calcium signaling to influence LV contractile behavior.
AC5 Protein Degradation

LV AC5 protein was barely detectable after AC6 deletion (Figure 2C) and was only detected after prolonged exposure in Western blotting. Although reduced AC5 protein synthesis may have contributed, cardiac AC5 mRNA levels were unchanged, and the primary mechanism appears to be increased proteasomal degradation of AC5 protein. Incubation of isolated cardiac myocytes from AC6−/− mice with MG132, a specific proteasomal inhibitor, increased AC5 protein content (Figure 2D). Increased protein degradation was not seen in 18 other key signaling proteins that were measured. The extent to which AC6 deletion alters stability of other proteins will require further study. It is noteworthy that targeted deletion of AC5 does not alter LV AC6 mRNA,9,11 and LV AC6 protein remains easily detectable.11 Thus, AC5 protein stability is governed by AC6. In contrast, deletion of AC5 does not appear to affect AC6 protein stability.9,11 Targeted disruption of caveolin-1 is associated with the disappearance of caveolin-2 and also is due to increased protein degradation,21 so the present finding does not stand in isolation.

Biological Role of AC6 Versus AC5

A goal in generating mice with AC6 deletion was to enable comparisons to mice with AC5 deletion, to identify unique roles of these 2 AC types on LV function. The unanticipated marked reduction in AC5 protein that accompanied AC6 deletion provided instead a functional dual knockout of AC6 and AC5. However, differences between mice with the functional AC6/AC5 deletion and mice with AC5 deletion reflect changes due to the AC6 deletion, and therefore, the strategy remains useful. For example, mice with the AC5 deletion have an unchanged cardiac reserve, but mice with the AC6 deletion (and consequent severe reduction in LV AC5 content) show marked impairment in cardiac reserve. It is interesting that LV function is not impaired even more, given that cardiac myocytes in these hearts have no AC6 and barely detectable levels of AC5. An important associated finding is that the functional deletion of AC6 and AC5 results in only a 70% reduction in LV cAMP-generating capacity, which indicates that (1) there remains enough AC5, even though not detectable by Western blotting, to provide a CAMP response, or (2) AC isoforms are present in cardiac myocytes other than AC5 and AC6 that contribute to CAMP generation. We previously reported the presence of AC2 mRNA using RNase protection assays in isolated adult porcine cardiac myocytes.3 AC isoform mRNA expression was unchanged by AC6 deletion, except for AC6 itself (Figure 2A and 2B). Unfortunately, the lack of antibodies capable of identifying low levels of AC isoforms (other than AC5/AC6) precludes measurement of protein content of these AC types.

The major AC isoforms in cardiac myocytes, AC5 and AC6, have 65% overall homology in amino acid sequence and are almost identical in intracellular catalytic domains. However, the deletion of AC6, compared with the deletion of AC5,9,11 yields striking differences in LV function, βAR signaling, and calcium handling, which indicates that the coexpression of these 2 AC types in cardiac myocytes does not represent biological redundancy. In some circumstances, AC5 deletion appears to have favorable10–12 and AC6 deletion unfavorable effects on the heart. For example, basal LV

Table 3. AC6 Deletion vs AC5 Deletion

<table>
<thead>
<tr>
<th></th>
<th>AC6 Deletion</th>
<th>AC5 Deletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>βAR-stimulated maximal +LV dP/dt</td>
<td>78% Decrease</td>
<td>No change</td>
</tr>
<tr>
<td>βAR-stimulated maximal −LV dP/dt</td>
<td>84% Decrease</td>
<td>No change</td>
</tr>
<tr>
<td>SERCA2a affinity for Ca2+</td>
<td>350% Decrease</td>
<td>33% Increase</td>
</tr>
<tr>
<td>PLN phosphorylation</td>
<td>46% Decrease</td>
<td>270% Increase</td>
</tr>
<tr>
<td>PKA activity</td>
<td>66% Decrease</td>
<td>No change</td>
</tr>
</tbody>
</table>

Studies were performed in our laboratory using AC5-deleted mice; these data were previously published11 and are listed here to enable a clear comparison with data from AC6-deleted mice in the present study, using identical assays performed by the same individuals. Percent changes indicate the changes of the specific AC deletion vs intact sibling controls for that line. *Decreased SERCA2a affinity for Ca2+ indicates an increase in EC50 and increased affinity indicates a decrease in EC50.

In conclusion, the combination of impaired cardiac cAMP generation and markedly disturbed calcium handling that result from deletion of AC6 underlies abnormalities in LV function. The biochemical and physiological consequences of function is increased and maximal LV function unchanged by AC5 deletion,11 but maximal LV function is decreased by AC6 deletion (Figure 4). These differences in the consequences of AC6 versus AC5 deletion on LV function have clear origins: Deletion of AC6 is associated with marked impairment of SR calcium handling, whereas AC5 deletion improves SR calcium handling (Table 3).11

Akt Activity

AC6 deletion was associated with diminished Akt phosphorylation at Ser473 in LV samples, an indication of reduced Akt activity (Figure 8). AC6 deletion was associated with sustained low levels of Akt phosphorylation, whereas intact mice showed reductions only when stimulated with βAR agonist. Increased Akt activity has been associated with reduced apoptosis in ischemia-reperfusion injury22 and also appears to promote cardiac hypertrophy.23 We are currently investigating whether AC6 deletion and reduced Akt activity might influence cardiac remodeling and apoptosis in pressure overload and myocardial infarction. In the present study, despite reduced Akt phosphorylation, we did not find changes in LV mass or in apoptosis. TUNEL staining indicated a very low occurrence of apoptotic cardiac myocytes (13 positives in 106 cardiac myocytes), except for young adult mice (<10 months old) with unstimulated, albeit dysfunctional hearts. It is noteworthy that AC5 deletion was found to be associated with reduced LV apoptosis rates, but this was evident only after LV pressure overload10 or in mice >20 months of age.12 Additional studies of the effects of AC6 deletion on apoptosis after superimposed cardiac stress and in the aging heart are being conducted but are beyond the scope of the present report. AC5 deletion is associated with increased longevity and progressive LV fibrosis in aged mice.12 Ongoing studies in our laboratory will determine whether AC6 deletion affects longevity and whether the LV dysfunction that we see in young adult mice progresses with age.

In conclusion, the combination of impaired cardiac cAMP generation and markedly disturbed calcium handling that result from deletion of AC6 underlies abnormalities in LV function. The biochemical and physiological consequences of
AC6 deletion reveal it to be an important effector molecule in the adult heart, serving unique biological functions not replicated by AC5.

Acknowledgments
We thank Diane Huang and Matthew Spellman for technical assistance.

Sources of Funding
This work was supported by National Institutes of Health grants P01 HL66941 and HL081741 (Dr Hammond), Merit Review Awards from the Department of Veteran’s Affairs (Drs Roth and Hammond), a Grant-in-Aid from the American Heart Association Western States Affiliate (Dr Gao), and a fellowship from the Banyu Life Science Foundation International (Dr Takahashi).

Disclosures
None.

References

CLINICAL PERSPECTIVE
Adenylyl cyclase (AC) content and function govern β-adrenergic receptor response and left ventricular contractility and therefore are of clinical interest. A fundamental understanding of the biochemical and physiological roles of AC is an indispensable first step in the development of new treatments for heart diseases. The specific roles and relative importance of AC6 versus AC5, the 2 AC types most abundantly expressed in cardiac myocytes, are unknown. Current understanding of the role of AC6 in cardiac function comes solely from gain-of-function models. To see directly the importance of AC6 in cardiac physiology and function, we generated mutant mice in which AC6 was absent. Deletion of AC6 has striking negative effects on the heart, effects that reverberate from the cellular to the organ level and include impairments in calcium handling, left ventricular force generation, and contractile responsiveness. These alterations are qualitatively and quantitatively different from those seen with AC5 deletion, which indicates that these 2 AC isoforms, which share substantial sequence homology, fulfill different biological roles. These results suggest that maintenance of AC6 content and function may be a rational therapeutic goal in heart failure, in which calcium handling, left ventricular force generation, and contractile responsiveness are impaired.
Adenylyl Cyclase Type 6 Deletion Decreases Left Ventricular Function via Impaired Calcium Handling

Tong Tang, Mei Hua Gao, N. Chin Lai, Amy L. Firth, Toshiyuki Takahashi, Tracy Guo, Jason X.-J. Yuan, David M. Roth and H. Kirk Hammond

Circulation. 2008;117:61-69; originally published online December 10, 2007; doi: 10.1161/CIRCULATIONAHA.107.730069

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/117/1/61

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2007/12/10/CIRCULATIONAHA.107.730069.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/