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

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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 β-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

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General agreement exists that the 2 adenylyl cyclase (AC) types most abundantly expressed in cardiac myocytes are types 5 (AC5) and 6 (AC6).[1–3] These 2 AC isoforms have extensive homology in amino acid sequence (65%), are each inhibited by micromolar concentrations of calcium, and respond similarly to β-adrenergic receptor (βAR) stimulation.[4,5] It has been speculated that coexpression in cardiac myocytes of 2 AC types may represent biological redundancy or, alternatively, that AC6 is predominant during development and AC5 predominates in the mature individual. Because they were recognized as pivotal AC isoforms in the heart >20 years ago,[1] it is surprising that the specific roles and relative importance of AC5 versus AC6, vis-à-vis cardiac function, remain to be established.

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AC5 and AC6 exhibit differences in their manner of regulation and effects on the failing heart. For example, AC6 mRNA is downregulated in pacing-induced congestive heart failure in pigs, but AC5 is not,[3] although both appear to be reduced in pacing-induced congestive heart failure in dogs.[1] When AC5 is expressed in cardiomyopathy (cardiac-directed Gαq expression), βAR-stimulated AC activity and cardiac function are increased, but hearts retain pathological hypertrophy and develop fibrosis.[6] In contrast, cardiac-directed expression of AC6 in this same model increases βAR-stimulated AC activity and cardiac function,[7] abrogates pathological hypertrophy (no fibrosis is observed), and prolongs life.[8] 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 crossbreeding 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

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Figure 1. Generation of AC6 gene-targeted mice. A, Schematic diagram of targeting strategy. 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. The 0.9-kb exon 1 is represented by a black box. B, Representative PCR analysis of tail genomic DNA from littermates generated by heterozygous pairs. The locations of PCR primers (P1 to P4) are shown in A. 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 *Stu* I-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.

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 failure[13,14] mandates studies of AC6 deletion, 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 *Sac*II-linearized targeting vector were selected with G418 for 10 days. Of 645 G418-resistant embryonic stem cell clones, 8 were identified as homologous recombinants by polymerase chain reaction (PCR) and Southern blotting. After confirmation of no chromosomal abnormality by karyotype examination, 2 of these clones were microinjected into C57BL/6 blastocysts, which were subsequently transferred to pseudopregnant females. Highly chimeric males were mated with C57BL/6 females, and heterozygous offspring were intercrossed to obtain homozygous mutant (AC6$^{-/-}$) mice. All experiments were performed with 6- to 10-month-old AC6$^{-/-}$ mice and intact littermates with normal AC6 expression (control). The present study was approved by the Animal Use and Care Committee of the VA San Diego Healthcare System, in accordance with Association for Assessment and Accreditation of Laboratory Animal Care guidelines.

Genotyping of Embryonic Stem Cells and Mice

Genomic DNA was extracted from embryonic stem cells and mouse tail clips. Two PCR reactions with primers specific for mutated allele

(P1: GGAGACCTAGAGATGGAGTG and P2: GCCACTTGTG-TAGCGCCAAG) and wild-type allele (P3: AAGATCTGCTTTGT-GGGTGC and P4: AGCCACTGGCTCGATTCGCGTGGCG) were used for genotype screening. Southern blotting subsequently was used for genotype confirmation. Briefly, genomic DNA was digested with *Stu*I, separated on a 0.75% agarose gel, transferred to a nylon membrane, and hybridized with a radiolabeled 0.4-kb probe outside the targeting construct region (Figure 1A). The probe hybridizes to a 5.3-kb fragment in the wild-type allele and to a 4.1-kb fragment in the mutated allele.

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) Ca^{2+} uptake was measured by the modified Millipore filtration technique as described previously.[16]

Measurements of Cytoplasmic Ca^{2+}

Cardiac myocytes were isolated from adult mice as described previously[11] and plated on laminin-coated 25-mm glass coverslips. Cells were loaded with the Ca^{2+}-sensitive fluorescent indicator Fura-2 AM (3 μmol/L) at 37°C for 30 minutes, with 5% CO$_2$ 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 CaCl$_2$, 1 mmol/L MgCl$_2$, 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+}]cyt) 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] Western blotting was performed as described previously.[16] Additional experiments were conducted with LV samples from isolated hearts perfused with dobutamine (10 μmol/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 β-mercaptoethanol, 50 mmol/L β-glycerophosphate, 1 mmol/L Na$_3$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 μmol/L proteasomal inhibitor MG132 (N-carbobenzyloxy-L-leucinyl-L-leucinyl-L-leucinal; Sigma, St. Louis, Mo) or lysosomal inhibitor E64 [trans-epoxysuccinyl-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 μm), 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 histopathologist unaware of group identity scored LV samples for the presence of inflammation and fibrosis.

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.

Table 1.   Morphometric Measurements

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

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.
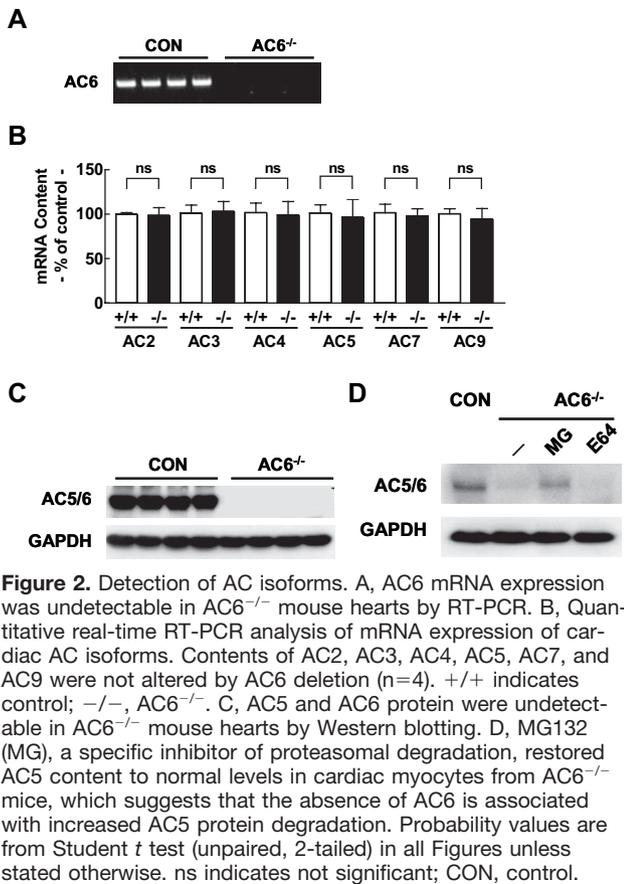
Results

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).

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

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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 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$^{-/-}$ mice, which suggests that the absence of AC6 is associated with increased AC5 protein degradation. Probability values are from Student t test (unpaired, 2-tailed) in all Figures unless stated otherwise. ns indicates not significant; CON, control.
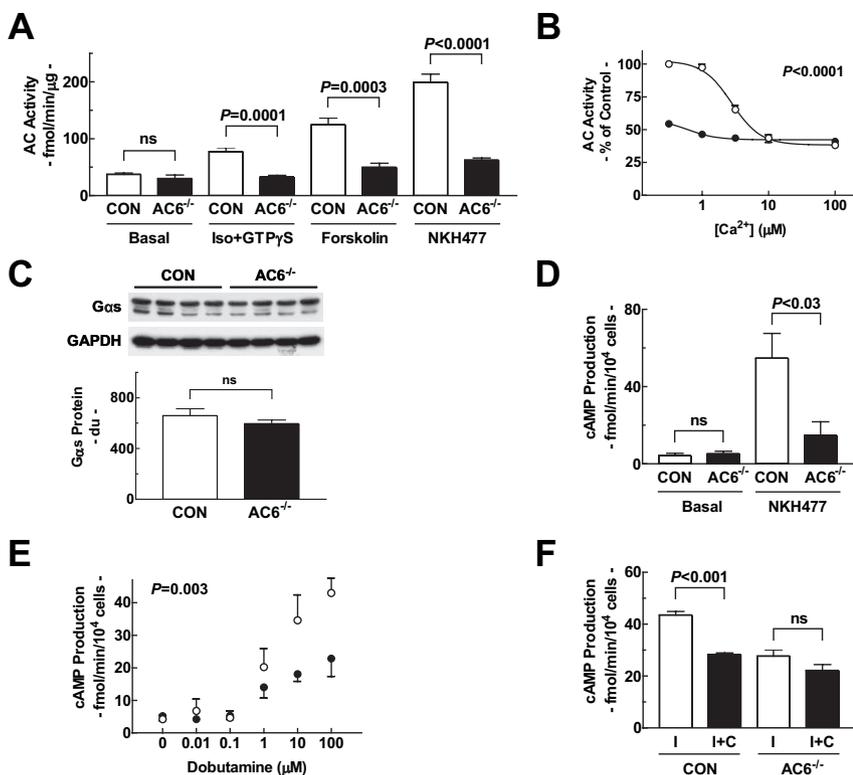
treatment was associated with increased AC5 protein content (Figure 2D). Treatment with E64, in contrast, did not change AC5 protein content (Figure 2D). These results indicate that

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, Gαs, Gαi, sarcoplasmic reticulum Ca^{2+}-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 GTPγS, 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 I, online-only Data Supplement). AC6 deletion impaired Ca^{2+}-mediated inhibition of AC activity (Figure 3B). Western blotting showed no change in LV protein contents of Gαs (Figure 3C) or Gαi (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

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Figure 3. A, AC6 deletion did not change basal cAMP, but stimulation with isoproterenol (Iso; 10 μmol/L) plus GTPγS (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 Ca^{2+}-mediated inhibition of AC activity in LV homogenates. Experiments were conducted in the presence of isoproterenol (10 μmol/L) and GTPγS (10 μmol/L; n=6). AC activity at 0.32 μmol/L free Ca^{2+} was set as 100%. C, AC6 deletion did not affect Gαs 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-deleted 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

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

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.

by 35% in normal cardiac myocytes, but cardiac myocytes from AC6⁻/⁻ mice did not show a significant reduction (Figure 3F).

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
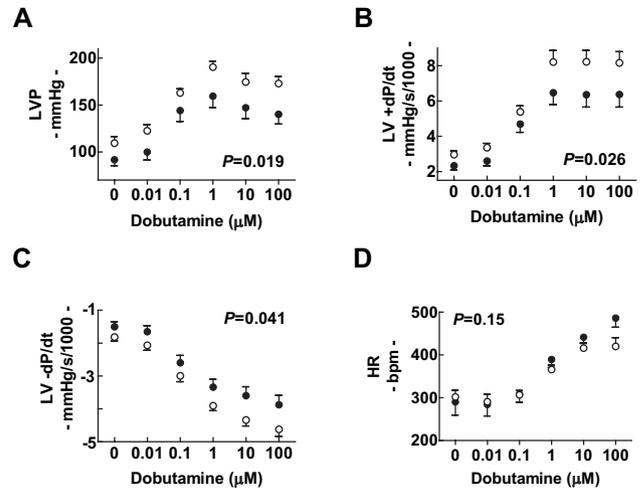
Figure 4. Cardiac responsiveness to βAR stimulation. A, AC6 deletion decreased LV developed pressure in isolated perfused hearts from control and AC6⁻/⁻ mice at various concentrations of dobutamine. LVP indicates LV pressure. B, AC6 deletion decreased LV+dP/dt in response to βAR stimulation. C, AC6 deletion 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⁻/⁻.

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⁻/⁻ mouse hearts 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 phosphorylated PLN in LV samples (Figure 5C and 5D). Similarly, AC6 deletion decreased protein content of Ser16 phosphorylated PLN in isolated mouse hearts perfused with 10 μmol/L dobutamine for 5 minutes (control 746±75 densitometric units [DU]; AC6⁻/⁻ 408±43 DU; $P=0.008$; n=4). Total protein content of PLN was unaltered by AC6 deletion (Figure 5C and 5E). In addition, protein contents of other βAR elements (eg, G-protein–coupled receptor kinase 2 [GRK2], GRK5, Gβ, Gγ, β₁AR, β₂AR, and muscarinic acetylcholine receptor [mAChR]), were not changed by AC6 deletion (Figure 5F).

To determine whether decreased PLN phosphorylation was mediated by protein phosphatase, we measured PP1 and PP2A activities in LV homogenates from AC6⁻/⁻ mice and their control littermates. AC6 deletion did not change protein phosphatase activities of PP1 (control 0.877±0.044 nmol · mg⁻¹ · min⁻¹; AC6⁻/⁻ 0.880±0.038 nmol · mg⁻¹ · min⁻¹; $P=0.98$; n=4) or PP2A (control 0.749±0.080 nmol · mg⁻¹ · min⁻¹; AC6⁻/⁻ 0.803±0.053 nmol · mg⁻¹ · min⁻¹; $P=0.71$; n=4). These results suggest that AC6 deletion decreases PLN phosphorylation by regulating PKA activity.

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. AC6 deletion was associated with decreased Ca²⁺ uptake at multiple Ca²⁺ concentrations (Figure 6A) and decreased SERCA2a affinity for Ca²⁺ (EC₅₀: control 0.76±0.03 μmol/L; AC6⁻/⁻
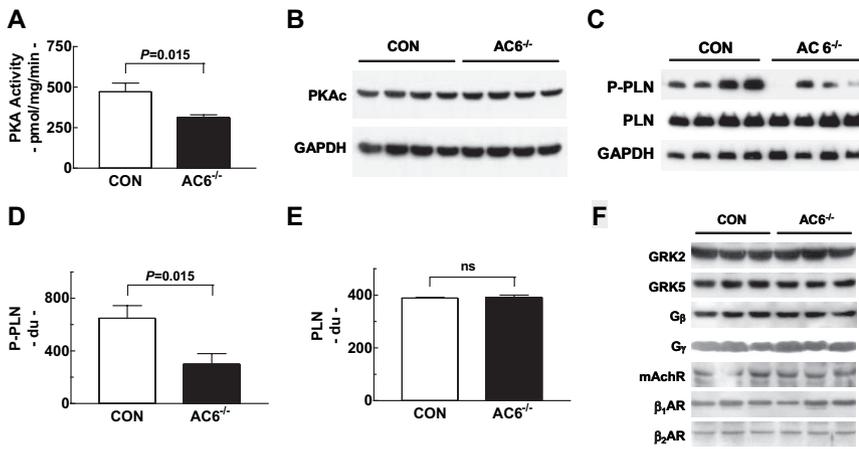
Figure 5. AC6 deletion decreased PKA activity and PLN phosphorylation. A, AC6 deletion was associated with decreased PKA activity (n=8). B, Representative Western blot showing normal PKA catalytic subunit content in LV samples from AC6$^{-/-}$ mice. C, Representative Western blot showing normal total PLN protein content and reduced Ser16 phosphorylated PLN content in LV samples from AC6$^{-/-}$ mice. D, Quantification of data from Western blotting showing AC6 deletion decreased Ser16 phosphorylated PLN content (n=8). E, Quantification of data from Western blotting showing AC6 deletion did not affect total PLN protein content (n=8). F, Representative Western blots showing that AC6 deletion did not affect LV levels of GRK2, GRK5, Gβ, Gγ, mAchR, β_1AR, and β_2AR. CON indicates control; PKAc, PKA catalytic subunit; du, densitometric units.

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 Ca^{2+} 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 Ca^{2+} Storage
To determine whether defective SR Ca^{2+} uptake that resulted from AC6 deletion could alter intracellular [Ca^{2+}], isolated cardiac myocytes were analyzed by real-time [Ca^{2+}]cyt imaging (Figure 7A). No difference was present between basal

intracellular Ca^{2+} levels in cardiac myocytes from AC6$^{-/-}$ mice and their intact control littermates (Figure 7B). However, caffeine-stimulated Ca^{2+} 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 Ca^{2+} uptake, indicate that cardiac myocyte SR Ca^{2+} 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/10^6 total myocytes; AC6$^{-/-}$ 13±38 apoptotic cells/10^6 total myocytes; $P=1.0$; n=4). There also were no group differences in
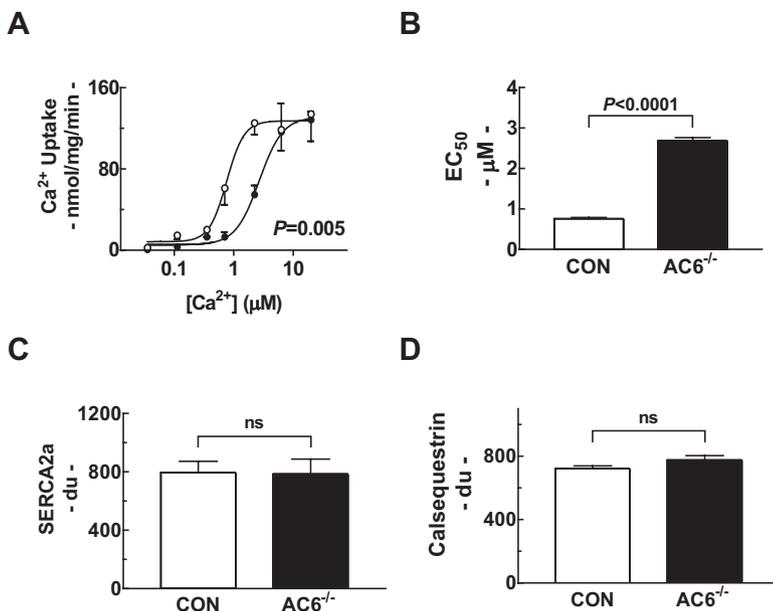

Figure 6. AC6 deletion impaired SR Ca^{2+} uptake. A, Initial rates of ATP-dependent Ca^{2+} uptake at different free calcium concentrations were measured in SR fractions of LV homogenates from AC6$^{-/-}$ mice and their intact siblings. Probability value shown is from 2-way ANOVA (control vs AC6$^{-/-}$); n=8. ○, control; ●, AC6$^{-/-}$. B, AC6 deletion decreased SERCA2a affinity for Ca^{2+} (n=8). EC$_{50}$ was calculated from the initial ATP-dependent Ca^{2+} uptake rate at different free Ca^{2+} concentrations. AC6 deletion did not affect LV protein content of SERCA2a (n=8; C) or calsequestrin (n=8; D). CON indicates control; du, densitometric units.

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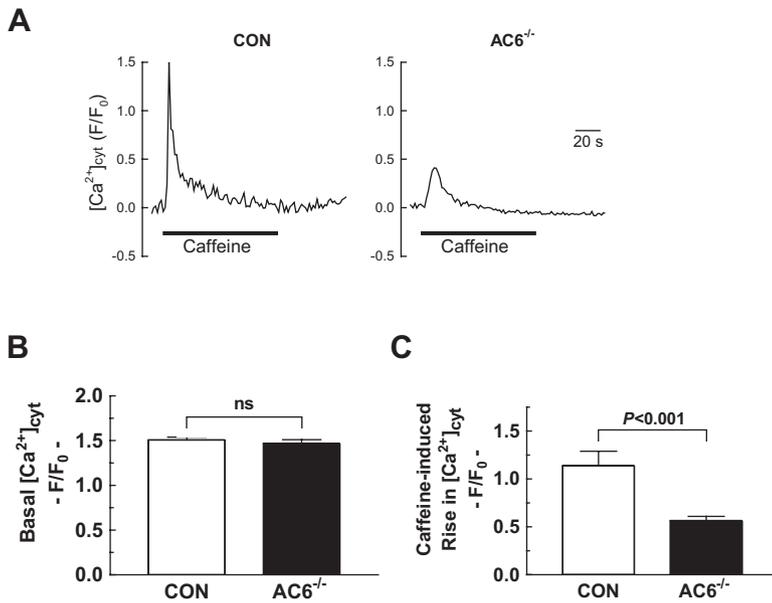
Figure 7. AC6 deletion decreased the peak amplitude of Ca^{2+} transients. A, Representative Ca^{2+} transients in response to caffeine stimulation were recorded with Fura-2 fluorescence. B, AC6 deletion did not affect basal $[Ca^{2+}]cyt$. C, AC6 deletion decreased transient peak amplitude in response to 10 mmol/L caffeine stimulation. Data for B and C were derived from 72 cells from 3 control mice (CON) and 59 cells from 4 $AC6^{-/-}$ mice.

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). Masson'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 quantita-

A

B

C

Figure 8. AC6 deletion decreased Akt phosphorylation. A, Representative Western blot showing unchanged total Akt protein content but reduced Akt phosphorylation at Ser473 in LV samples from $AC6^{-/-}$ mice. B, Quantification of data from Western blotting showing that AC6 deletion decreased Ser473 phosphorylated Akt content (n=8) but did not alter total Akt protein content (n=8; C). du indicates densitometric units.

tively different from those seen in AC5 deletion, they are often directionally opposite. For example, LV calcium handling was improved by AC5 deletion[11] 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 Ca^{2+} 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 cavelolin-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 favorable[10–12] and AC6 deletion unfavorable effects on the heart. For example, basal LV

Table 3.   AC6 Deletion vs AC5 Deletion

	AC6 Deletion	AC5 Deletion[11]
βAR-stimulated maximal +LV dP/dt	78% Decrease	No change
βAR-stimulated maximal −LV dP/dt	84% Decrease	No change
SERCA2a affinity for Ca^{2+}*	350% Decrease	33% Increase
PLN phosphorylation	46% Decrease	270% Increase
PKA activity	66% Decrease	No change

Studies were performed in our laboratory using AC5-deleted mice; these data were previously published[11] 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 Ca^{2+} indicates an increase in EC_{50}, and increased affinity indicates a decrease in EC_{50}.

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 injury[22] 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 10^6 cardiac myocytes), and LV caspase 3/7 activity was low and showed no group differences, which provides additional support that LV apoptosis is not activated in these young adult mice (<10 months old) with unstressed, 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 overload[10] 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.

Disclosures
None.

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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 β-adrenergic receptor signaling, 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.

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