Adenylylcyclase Increases Responsiveness to Catecholamine Stimulation in Transgenic Mice

Mei Hua Gao, PhD; N. Chin Lai, PhD; David M. Roth, MD, PhD; Jinyao Zhou, BS; Jian Zhu, BS; Toshihisa Anzai, MD; Nancy Dalton, BA; H. Kirk Hammond, MD

Background—The cellular content of cAMP generated by activation of adenylylcyclase (AC) through the β-adrenergic receptor (βAR) is a key determinant of a cell’s response to catecholamine stimulation. We tested the hypothesis that increased AC content, independently of βAR number, increases responsiveness to catecholamine stimulation in vivo.

Methods and Results—Transgenic mice with cardiac-directed expression of ACVI showed increased transgene AC expression but no change in myocardial βAR number or G-protein content. When stimulated through the βAR, cardiac function was increased, and cardiac myocytes showed increased cAMP production. In contrast, basal cAMP and cardiac function were normal, and long-term transgene expression was not associated with abnormal histological findings or deleterious changes in cardiac function.

Conclusions—The amount of AC sets a limit on cardiac β-adrenergic signaling in vivo, and increased AC, independent of βAR number and G-protein content, provides a means to regulate cardiac responsiveness to βAR stimulation. Overexpressing an effector (AC) does not alter transmembrane signaling except when receptors are activated, in contrast to receptor/G-protein overexpression, which yields continuous activation and has detrimental consequences. Our findings establish the importance of AC content in modulating β-adrenergic signaling in the heart, suggesting a new target for safely increasing cardiac responsiveness to βAR stimulation. (Circulation. 1999;99:1618-1622.)

Key Words: receptors, adrenergic, beta proteins adenylcyclase

Adenylylcyclase Increases Responsiveness to Catecholamine Stimulation in Transgenic Mice

A denosine 3′,5′-cyclic monophosphate (cAMP) is a pivotal second messenger that regulates cellular function. A large number of agonists act via membrane receptors and GTP-binding proteins (such as the stimulatory GTP-binding protein, Gs) to regulate cAMP generation via adenylylcyclase (AC). β-Adrenergic receptors (βARs) are key targets of agonists that promote cAMP generation in a wide variety of cells and are particularly important in cardiac myocytes, in which they regulate cardiac contraction and relaxation as well as heart rate.

The estimated molar proportions of the elements of the βAR/G/AC complex in cardiac myocytes are 1:200:3,1 suggesting that βAR number or the amount of AC may limit βAR-mediated transmembrane signaling. Increasing βAR number 20- to 200-fold in cultured cardiac myocytes2 and transgenic mice3 achieved only 2-fold increases in cAMP production. Similarly, cardiac-directed overexpression of Gsα in transgenic mice only minimally increased cardiac adrenergic responsiveness.4 A fixed amount of AC may have limited the degree to which increased βAR or Gsα could provoke a substantial increase in second messenger generation and physiological response. Indeed, in 1969 it was suggested that AC may limit cellular responsiveness to agonist stimulation,5 and recent studies showed that increased expression of AC in cultured cardiac myocytes yields a proportional increase in cAMP production.6 These studies underscore the importance of AC in transmembrane signaling but do not establish that AC content governs adrenergic responsiveness in vivo. Furthermore, previous studies that overexpressed the βAR in the heart found that heart rate, function, and cAMP generation were increased, even in the unstimulated state.3 It was concluded that sustained β-adrenergic stimulation resulted in cardiomyopathy and substantial cardiac histological abnormalities in hearts of older transgenic mice overexpressing cardiac Gsα.7 Similar deleterious consequences of cardiac-directed overexpression of βARs have been reported.8 Here we test the hypothesis that increasing the expression of the effector (AC) will increase the responsiveness of the heart to βAR stimulation and that increasing the amount of AC, without changing βAR number or Gsα, provides a means for the same number of receptors to transduce signals more effectively in vivo. We thought that increasing AC without altering cell-surface βAR number or G-protein content might elude the potentially deleterious effects of sustained adrenergic stimulation.

Received August 17, 1998; revision received October 10, 1998; accepted November 23, 1998.

From the Departments of Medicine, VAMC-San Diego and University of California San Diego, and Department of Anesthesiology (D.M.R.), University of California San Diego, La Jolla, Calif.

Drs Lai and Roth and Mr Zhou contributed equally to this study.

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

© 1999 American Heart Association, Inc.

Circulation is available at http://www.circulationaha.org

1618
Longer exposure (5 minutes) reveals endogenous AC VI d, different lines, despite variations in AC mRNA expression. Levels of transgene protein expression were similar among different-duration exposure (1 minute). Each lane was loaded with equal amounts of total protein from 3 animals.

**Methods**

### Generation of Transgenic Mice

The use of animals was in accordance with institutional guidelines. A 10.8-kb fragment containing the expression cassette of the construct (Figure 1a) was used for microinjection. Sixteen of 70 offspring were positive for the transgene, as shown by polymerase chain reaction (PCR) of the tail tip (confirmed by Southern blotting; offspring were positive for the transgene, as shown by polymerase chain reaction (PCR) of the tail tip (confirmed by Southern blotting; founder animals were crossbred with wild-type mice of the same strain, and selected animals were killed for analysis of cardiac transgene expression reverse-transcription (RT)-PCR (for quantitative assessment of the level of expression; data not shown), Northern blotting, and Western blotting (Figure 1b and 1c). AC VI mRNA expression levels were markedly increased but variable (Figure 1b). Even so, AC VI protein expression (Figure 1c and 1d) was constant, and lines with similar AC VI protein expression were used for the study, with transgene-negative siblings serving as controls. Litter size was normal, and mortality by 19 months was rare and similar between transgene-positive and control animals.

### Documentation of Transgene Expression

Total RNA was extracted by homogenization of tissue in 2 mL of RNA STAT-60 (Tel-Test Inc.). Denatured total RNA (20 μg) was electrophoresed in 1× MOPS/EDTA buffer on a 1.0% agarose gel. RNA was transferred onto a nylon membrane in 20× SSC solution, immobilized (80°C, 2 hours), and hybridized with a [32P]dCTP-labeled murine AC VI cDNA probe. RT-PCR was used to estimate the quantities of transgene AC VI mRNA (Figure 1). Total RNA (1 μg) was mixed with 1 μg of transgene-specific primer. The RT reaction was carried out by use of the Superscript II kit and instruction (Gibco-BRL Life Technologies). RT product (5 μL) was used as template for PCR with transgene-specific primers (indicated as T1 and T2 in Figure 1).

To document transgene protein expression, a polyclonal antibody recognizing AC VI and AC VI protein (Santa Cruz Biosciences) was used in Western blots conducted on cardiac homogenates. Left ventricular (LV) samples were homogenized in 1 mL cold Tris buffer (25 mmol/L Tris HCl, 25 mmol/L Tris Base, 8 mmol/L MgCl2, and 0.5 mmol/L EGTA, pH 7.5). Protease inhibitors were added (final concentrations, in μg/mL: leupeptin 10, aprotinin 10, pepstatin 10, and 4-[2-aminomethyl]benzenesulfonyl fluoride [Pefabloc] 100), followed by centrifugation (40 000g, 20 minutes, 4°C). The pellet was resuspended in Tris buffer and sonicated. For Western blot analysis, 100 μg protein was separated on 7.0% PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with anti-AC VI/Ac VI antibody diluted 1:100 in blocking buffer (5% nonfat dry milk in Tris buffer; 0.9% NaCl, 10 mmol/L Tris HCl, pH 7.5). Primary antibodies were detected with goat anti-rabbit IgG horseradish peroxidase conjugate (Gibco-BRL Life Technology) in blocking buffer. The antigen was visualized with chemiluminescent substrates A and B (Kirkegaard and Perry Laboratories) and exposed to x-ray film.

### Echocardiography

Echocardiography was performed in anesthetized mice as previously reported. Animals were anesthetized with ketamine 100 mg/kg IP and xylazine 5 mg/kg IP. An echocardiograph (Interspec Apogee CX) with dynamically focused symmetrical annular array technology for 2-dimensional and M-mode imaging was used (9-MHz transducer). With mice in the left lateral decubitus position, a parasternal short-axis view was obtained as a guide for LV M-mode imaging at the papillary muscle level. M-mode tracings were recorded on videotape. Pulsed Doppler images of estimated peak LV outflow velocity and mitral inflow velocities were obtained in a modified parasternal long-axis view. The M-mode images were digitized and visualized with a scanner to facilitate accurate measurement. The images were measured with NIH Image (version 1.52) on a computer screen with leading-edge techniques. Chamber dimensions and the velocity of circumferential fiber shortening were obtained. Data were acquired and analyzed without knowledge of whether animals were transgene-positive or controls.

### In Vivo Physiology

Mice were anesthetized with ketamine 50 mg/kg IP and thiobutabarbital 100 mg/kg IP (Inactin, Research Biochemical International). A cervical incision was made and the trachea intubated and connected to a volume-cycled ventilator. A 1.4F micrometer catheter (Millar Instruments) was inserted via the right carotid artery and advanced into the LV. The left carotid artery was cannulated with flame-stretched PE 50 tubing and connected to a low-compliance pressure transducer (Abbott Laboratories). Bilateral vagotomy was performed to minimize confounding effects of reflex activation during agonist infusion. The left external jugular vein was cannulated with flame-stretched PE 50 tubing and connected to a low-compliance pressure transducer. In Vivo Physiology

Cardiac function was determined in response to adrenergic stimulation in isolated perfused hearts with an intraventricular balloon catheter to determine isovolumic LV pressure as recently described. Mice were anesthetized with ketamine 100 mg/kg IP and xylazine 5 mg/kg IP, and heparin was given (5000 IU/kg IP). Hearts were excised after midline sternotomy. The aortic root was cannulated with a 22-gauge cannula and suspended in an isolated perfused heart apparatus. Retrograde perfusion of the heart was established with warmed Krebs-Henseleit solution oxygenated with 95% O2 and 5% CO2. The hearts were perfused at a constant pressure (80 mm Hg) while a polyethylene balloon was placed in the LV through a small incision in the left atrium. The fluid-filled balloon was connected via 2 lumens to a 100-μL syringe for filling the balloon and a 1.8F high-fidelity pressure transducer for the measure-
ment of LV pressure. After cannulation and balloon placement, the hearts were allowed to equilibrate for 10 minutes. Baseline heart rate and LV pressure development were recorded. The hearts were then perfused with isoproterenol in bolus doses equal to final concentrations of 0.1 to 1000 μmol/L at 5-minute intervals. LV developed pressure was recorded for 10 sequential beats at the peak of the response. Data were recorded on disk and analyzed as above. Peak rates of LV pressure development (LV dP/dt) and relaxation (LV –dP/dt) were calculated after acquisition at a sampling rate of 3000 per second (Dataq DI-400, Windaq Software).

Isolation of Cardiac Myocytes and cAMP Generation

LV myocytes were isolated by use of modifications of methods previously described.12 Mice were heparinized (5000 IU/kg) and anesthetized with ketamine 100 mg/kg and xylazine 5 mg/kg IP, hearts were removed, and aortas were cannulated and connected to a perfusion apparatus. Hearts were perfused for 3 minutes (2 ml/min) with calcium-free medium containing Joklik-modified minimal essential medium (Gibco-BRL) with final concentrations (mmol/L) of NaCl 113, KCl 4.7, MgCl2 1, NaHPO4 2H2O 8.4, glucose 20, NaHCO3 12, KHCO3 10, Na-HEPES 10, taurine 30, creatinine 2 and creatine (pH 7.36). The perfusion medium then was switched to the above solution with collagenase (Worthington Type II, 33 IU/ml, 1 mg/ml) for 20 minutes, and hearts were removed from the apparatus. Atria were removed and ventricles cut into small pieces and triturated with a wide-bore pipette in collagenase solution (10 ml for 10 minutes). Free myocytes were centrifuged twice (400 rpm for 2 minutes) and washed in 1% BSA. Before treatment of cells, growth medium was removed and cells were equilibrated for 30 minutes (25°C) in serum-free and NaHCO3-free DMEM supplemented with 20 mmol/L HEPES (pH 7.2).

Equal numbers of viable cardiac myocytes were incubated (10 minutes, 25°C) in fresh DMEM containing no addition (basal), isoproterenol 10 μmol/L, or forskolin 10 μmol/L. Aspiration of medium and addition of 7.5% ice-cold trichloroacetic acid (TCA) terminated the reaction. TCA extracts were frozen (−20°C) until assayed. Intracellular cAMP levels were determined by radioimmunoassay (Amersham Life Science).

βAR Number and G-Protein and G-Protein Receptor Kinase Content

βARs were estimated in radioligand-binding experiments using [125I]iodocyanopindolol 212 pmol/L; binding in the presence of 10−4 mol/L isoproterenol defined nonspecific binding.6 Experiments were performed on triplicate samples. Polyclonal antibodies recognizing Gβα, Gαs and G-protein receptor kinase types 2 (GRK2) and 5 (GRK5) were used in Western blots conducted on cardiac homogenates as previously described.13

Results

Transgenic Mice

Using standard procedures for the generation of transgenic mice, we obtained substantial and selective cardiac expression of ACVI, documented by PCR, RT-PCR, Northern blotting, and immunoblotting (Figure 1). ACVI mRNA was increased 10- to 80-fold in hearts from transgene-positive animals, and ACVI protein was consistently increased 20-fold in hearts of animals from these lines. No mRNA was detected in other tissues by Northern blot analysis (data not shown). Body weight (control: 31±2 g, n = 23; transgene: 30±1, n = 27), tibial length (control: 18.4±0.2 mm, n = 11; transgene: 18.2±0.2, n = 18), and right ventricular and LV wet weight (control: 105±6 mg, n = 23; transgene: 102±4 mg, n = 27) and dry weight (control: 28±2 mg, n = 9; transgene: 28±1 mg, n = 12) were unchanged by transgene expression. Histological assessment showed no fibrosis or other abnormalities in the heart or other organs, even in 15- and 19-month-old animals (n = 3, data not shown).

Echocardiography: Heart Size and Function

<table>
<thead>
<tr>
<th></th>
<th>Control (n)</th>
<th>TG (n)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>231±27 (7)</td>
<td>257±16 (13)</td>
<td>0.39</td>
</tr>
<tr>
<td>Velocity of circumferential fiber shortening, circ/s</td>
<td>4.82±0.27 (7)</td>
<td>4.88±0.31 (13)</td>
<td>0.90</td>
</tr>
<tr>
<td>End-diastolic diameter, mm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At 4 months</td>
<td>3.99±0.10 (7)</td>
<td>3.75±0.20 (13)</td>
<td>0.20</td>
</tr>
<tr>
<td>At 21 months</td>
<td>3.79±0.28 (5)</td>
<td>3.73±0.25 (7)</td>
<td>0.70</td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>33±1 (7)</td>
<td>37±2 (13)</td>
<td>0.18</td>
</tr>
<tr>
<td>At 21 months</td>
<td>32±3 (5)</td>
<td>36±5 (7)</td>
<td>0.21</td>
</tr>
</tbody>
</table>

TG indicates transgene-positive animals. Values are mean±SEM. P values are from Student’s t test (unpaired, 2-tailed).

In Vivo Physiology

Stimulation of the heart in vivo with the βAR agonist isoproterenol showed that these phenotypically normal hearts responded to adrenergic stimulation in a unique manner (Figure 2). LV +dP/dt and −dP/dt as well as heart rate and systolic blood pressure (Figure 2) were increased in hearts of

> Figure 2. Cardiac responsiveness to adrenergic stimulation in vivo. To assess effect of cardiac-directed expression of ACVI on cardiac function in vivo, we measured LV +dP/dt (a) and LV −dP/dt (b), heart rate (HR, c), and systolic blood pressure (SBP, d) in response to injections of (−)-isoproterenol. Probability values are from 2-way ANOVA. *, Mean values from 5 control animals; ●, from 5 transgene-positive animals. In graphs in all figures, error bars denote 1 SEM.
transgene-positive animals, indicating that increased AC content augments cardiac responsiveness to cAMP stimulation. We also assessed the effects of a water-soluble forskolin derivative (NKH447; see Reference 10), which directly stimulates AC without interacting with the βAR, and again found that transgene-positive animals exhibited increased rates of LV +dP/dt (control: 5790±450 mm Hg/s, n=4; transgene: 12 982±2720 mm Hg/s, n=5; P=0.028) and LV −dP/dt (control: −5722±135 mm Hg/s, n=4; transgene: −12 362±1263 mm Hg/s, n=5; P<0.001) as well as heart rate (control: 440±13 bpm, n=4; transgene: 557±36 bpm, n=5; P=0.028).

Ex Vivo Physiology
When hearts of transgene-positive and control mice were isolated from neural input and the circulation, they showed similar basal intrinsic heart rates (control: 364±25 bpm, n=6; transgene: 408±19 bpm, n=11; P=0.12) and basal LV dP/dt (control: 2860±325 mm Hg/s, n=6; transgene: 3189±263 mm Hg/s, n=11; P=NS) (Figure 3). However, LV dP/dt in response to isoproterenol stimulation was increased in transgene-positive animals through a wide range of isoproterenol concentrations (P<0.0001, Figure 3), even though heart rate was unchanged (Figure 3). LV end-diastolic pressures were stable throughout the studies and were not different between groups (control: 13±1 mm Hg; range, 12 to 13 mm Hg; transgene: 12±1 mm Hg; range, 11 to 13 mm Hg).

Transmembrane βAR Signaling
Cardiac myocytes from transgene-positive animals showed increased cAMP production when stimulated by forskolin and isoproterenol (Figure 4a). These data document that cardiac myocytes expressing transgene ACVI have increased adrenergic responsiveness not only to direct stimulation of AC by forskolin, reflecting increased amounts of AC, but also to isoproterenol, indicating that increased AC is functionally coupled and recruitable through βAR stimulation.

To determine whether increased AC expression, with attendant alterations in cAMP production, might have affected the expression of other elements in the βAR transmembrane signaling cascade,14 we assessed myocardial βAR number, cardiac G-protein content, and GRK2 and GRK5 (predominant GRK isoforms in mammalian heart15). GRK uncouples the βAR from Gαi, attenuating signal transduction. Radioligand binding assay and immunoblotting indicated similar βAR number and Gαi, and Gαi2 content in hearts from transgene-positive and control animals (Figure 4b and 4c). In contrast, GRK2 content (but not GRK5) was increased (Figure 4d).

Discussion
To explore the influence of increased AC content on heart function and transmembrane signaling, we overexpressed ACVI, a major AC isoform in cardiac myocytes,13 in transgenic mice. This allowed us to assess the effects of variations in myocardial ACVI content on cardiac structure and function in vivo and facilitated detailed studies of adrenergic signaling in isolated cardiac myocytes. The relationships between myocardial AC content, signal transduction, and heart function were our focus.

Despite increased cardiac AC expression, basal heart rate and contractile function were unchanged when examined by echocardiography (Table). Basal cAMP generation in cardiac myocytes from transgene-positive mice also was unchanged (Figure 4). These data indicate that basal cardiac βAR responsiveness is unaltered in transgene-positive animals.
Why then were the LV dP/dt and heart rate increased in the unstimulated state (0 isoproterenol, Figure 2)? Measurement of LV dP/dt required surgical intervention and mechanical ventilation, which increase catecholamine release.\(^5\) In this setting, higher LV dP/dt and heart rates in transgene-positive animals most likely reflect increased responsiveness to elevated endogenous catecholamines. To determine whether this was in fact the case, we isolated hearts of transgene-positive and control mice from neural input and the circulation, and they showed similar intrinsic heart rates but increased LV dP/dt in response to adrenergic stimulation at matched heart rates (Figure 3). The noninvasive echocardiographic studies did not require surgery or mechanical ventilation and are in closer accord with the unstimulated state.

A 2.1-fold increase in GRK2 content makes the increase in βAR-stimulated cAMP generation even more impressive and may help to explain why basal cAMP generation and cardiac function are unchanged despite enhanced responsiveness of both to βAR stimulation. Thus, increased adrenergic signaling results from increased ACVI content, not from alterations in other elements in the βAR signaling pathway, and additional ACVI was coupled to endogenous βARs. These results indicate that increasing the expression of the effector (AC) can influence the responsiveness of a cell to βAR stimulation without changing the amount of the receptor or G protein.

Augmentation of transmembrane adrenergic signaling by increasing cardiac βAR or Gs expression\(^2 - 4\) or inhibiting GRK function\(^8\) achieves no more than a 2-fold increase in cAMP production. In contrast, the present study indicates that overexpression of ACVI in cardiac myocytes is associated with a robust amplification of βAR-mediated signaling (2.7-fold), despite unchanged βAR and G-protein expression and increased GRK2. It appears that AC holds a pivotal position in transmembrane signaling and is the limiting factor governing intracellular cAMP generation in response to neurohumoral adrenergic stimulation.

Cardiac-directed expression of AC results in anatomically normal hearts with normal basal function, and there is no decline in function in older mice (Table); myocardial fibrosis is not present even in 19-month-old animals. This is in contrast to cardiac-directed overexpression of Gs or βARs, which results in dilated cardiomyopathy and cardiac fibrosis as animals age.\(^7,8\) The disparities between previous models and the present study with regard to cardiac structure and function suggest an intrinsic difference between receptor/transducer versus effector amplification. This is likely to be that overexpression of βAR and Gs (but not AC) results in sustained βAR activation,\(^3,7\) which, ultimately, has detrimental consequences.\(^7,8\)

In conclusion, we have shown that transgenic mice with cardiac-directed expression of ACVI have structurally normal hearts with normal basal function. Cardiac responsiveness to adrenergic stimulation is increased, with amplified transmembrane signaling and increased physiological function. Cardiac myocytes isolated from transgene-positive hearts respond to adrenergic stimulation with increased cAMP production. These data indicate that the amount of AC sets a limit on cardiac β-adrenergic signaling in vivo and that increased AC, independent of βAR number and G-protein content, provides a means to regulate cardiac responsiveness to adrenergic stimulation. Our findings establish the importance of AC content in modulating β-adrenergic signaling in the heart and potentially for other AC-linked receptors in other cells. Our data suggest a potential target for increasing cardiac responsiveness to adrenergic stimulation.

Acknowledgments

This research was supported by a Merit Award from the Department of Veteran’s Affairs (Dr Hammond) and Specialized Center of Research on Congestive Heart Failure 1 P50 HL-53773-01 (Dr Hammond). Additional support was provided by Institutional National Research Service Award HL-07444 (Dr Gao) and a grant from the Foundation for Anesthesiology Education Research/Society of Cardiovascular Anesthesiologists (Dr Roth). Portions of this research were supported by an unrestricted grant from Collateral Therapeutics, Inc, San Diego, Calif, for which Dr Hammond consults. The authors thank Dr Jeffrey Robbins for providing the α-myosin heavy chain promoter; Nippon Kayaku Co, Ltd, for providing NIK477; Dr Robert Ross for helpful comments; and Drs Tamsin Lisa Kelly and Paul A. Insel for reviewing the manuscript.

References

Adenylylcyclase Increases Responsiveness to Catecholamine Stimulation in Transgenic Mice
Mei Hua Gao, N. Chin Lai, David M. Roth, Jinyao Zhou, Jian Zhu, Toshihisa Anzai, Nancy Dalton and H. Kirk Hammond

_Circulation_. 1999;99:1618-1622
doi: 10.1161/01.CIR.99.12.1618
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 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/99/12/1618

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/