Adenylylcyclase Increases Responsiveness to Catecholamine Stimulation in Transgenic Mice

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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 or 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 | adenylylcyclase

A
denose 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, Gi) 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 200-fold increased cAMP production. Similarly, cardiac-directed overexpression of Giα in transgenic mice only minimally increased cardiac adrenergic responsiveness. A fixed amount of AC may have limited the degree to which increased βAR or Gi 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, and recent studies showed that increased expression of AC in cultured cardiac myocytes yields a proportional increase in cAMP production. 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. It was concluded that sustained β-adrenergic stimulation resulted in cardiomyopathy and substantial cardiac histological abnormalities in hearts of older transgenic mice overexpressing cardiac Giα. Similar deleterious consequences of cardiac-directed overexpression of βARs have been reported. 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 Gi, 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.
Western blot analysis. ACVI protein was barely detectable in cardiac homogenates. In contrast, hearts from transgene-positive animals (t) showed abundant AC VI protein. Levels of transgene protein expression were similar among different lines, despite variations in AC mRNA expression. c and d, Longer exposure (5 minutes) reveals endogenous ACVI; d, shorter-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; data not shown). Founder animals were crossbred with wild-type mice of the same strain, and selected animals were killed for analysis. Litter size was normal, and mortality by 19 months was rare and similar between transgene-positive and control animals. Mortality was due to heart failure (see Ex Vivo Physiology).

Ex 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 previously described.11 Mice were anesthetized with ketamine 100 mg/kg IP and xylazine 5 mg/kg IP. An echocardiograph (Interspec Apogee CX) was placed on the chest. Echocardiography was performed in anesthetized mice as previously reported.9 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 recordings were made with a camera 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.9 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.
Isolation of Cardiac Myocytes and cAMP Generation

LV myocytes were isolated by use of modifications of methods previously described. 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, NaH2PO4 1.4, glucose 20, NaHCO3 12, KHCO3 10, Na-HEPES 10, taurine 30, carnitine 2, and creatine 2 (pH 7.36). The perfusion medium then was switched to the above solution with collagenase (Worthington Type II, 33 IU/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 7.4). Parallel cultures were equilibrated in serum-containing DMEM containing 20% FBS and 1 ng/mL recombinant human interleukin-1β.

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 Gβγ, 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±28 (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>At 4 months 3.99±0.10 (7)</td>
<td>3.75±0.20 (13)</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>At 21 months 3.79±0.28 (5)</td>
<td>3.73±0.25 (7)</td>
<td>0.70</td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>At 4 months 33±1 (7)</td>
<td>37±2 (13)</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>At 21 months 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
transgene-positive animals, indicating that increased AC content augments cardiac responsiveness to catecholamine stimulation. We also assessed the effects of a water-soluble forskolin derivative (NKH47; 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, we assessed myocardial βAR number, cardiac G-protein content, and GRK2 and GRK5 (predominant GRK isoforms in mammalian heart13). GRK uncouples the βAR from Gi, attenuating signal transduction. Radioligand binding assay and immunoblotting indicated similar βAR number and Gαi1 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 was also unchanged (Figure 4). These data indicate that basal cardiac βAR responsiveness is unaltered in transgene-positive animals.

Figure 3. Cardiac responsiveness to adrenergic stimulation ex vivo. To assess effect of cardiac-directed expression of ACVI on cardiac function independent of potentially confounding influences of autonomic reflex activation and endogenous catecholamines, we performed studies on isolated, perfused hearts. In these studies, we measured LV dP/dt (a) and LV –dP/dt (b), heart rate (HR, c), and LV pressure (LVP, d) in response to injection of 10 μmol/L isoproterenol. Probability values are from 2-way ANOVA. ○, Mean values from 6 control animals; ●, from 11 transgene-positive animals. LV +dP/dt and LV –dP/dt and LVP were increased in hearts from transgene-positive mice. Heart rate was unchanged.

Figure 4. a, Cardiac myocyte responsiveness to isoproterenol. Cardiac myocytes from transgene-positive animals showed increased cAMP production when stimulated by 10 μmol/L forskolin (Fsk) and 10 μmol/L isoproterenol (Iso), but basal cAMP production was unaltered. #P = 0.009, ##P = 0.029, Student’s t test for unpaired data, 2-tailed; transgene-negative, n = 5; transgene-positive, n = 6. b, β-AR number. Myocardial β-AR number, estimated in radioligand binding experiments, was not different between control (Con) and transgene-positive (TG) animals; n = 4 for both groups. c, G-protein content. Myocardial G-protein content (Gsα and Gβγ), estimated in immunoblotting experiments, was not different between control (open bars) and transgene-positive (hatched bars) animals; n = 7 for each group. d, GRK content. Cardiac-directed expression of ACVI had no effect on immunodetectable GRK5 (data not shown), but cardiac GRK2 protein content was increased 2.1-fold in transgene-positive (hatched bars) animals; n = 5 for each group.
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. In this
setting, higher LV dP/dt and heart rates in transgene-positive
animals most likely reflect increased responsiveness to ele-

vated 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 signal-
ning resulted from increased ACVI content, not from alterations
in other elements in the βAR signaling pathway, and addi-
tional 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 expression2–4 or inhibiting
GRK function16 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 govern-
ing intracellular cAMP generation in response to neuro-
mural 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 amplifi-
cation. 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 transmem-
brane 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 respon-
siveness to adrenergic stimulation.

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