Diminished β-Adrenergic Receptor Responsiveness and Cardiac Dilation in Hearts of Myopathic Syrian Hamsters (BIO 53.58) Are Associated With a Functional Abnormality of the G Stimulatory Protein

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Previous studies have demonstrated a diminution in the bioactivity of the guanine nucleotide–binding regulatory protein that stimulates adenylyl cyclase (G<sub>s</sub>) in hearts of the hypertrophic BIO 14.6 Syrian hamster. In this study, we measured functional activity and immunodetectable levels of G<sub>s</sub> in a mutant strain of hamsters (BIO 53.58) that develop a dilated cardiomyopathy. Pathological studies demonstrated that 100-day-old BIO 53.58 hamsters had substantial ventricular dilation when compared with age-matched F<sub>1</sub>B controls. Additionally, these 100-day-old hamsters demonstrated diminished contractile response to β-adrenergic receptor stimulation. The pathological and hemodynamic changes were associated with defective coupling of G<sub>s</sub> to adenylyl cyclase as adenylyl cyclase activation was distinctly decreased in the presence of isoproterenol, fluoride ion, guanine nucleotides, and forskolin. Additionally, the ability of the α-subunit of G<sub>s</sub> to reconstitute isoproterenol-stimulated adenylyl cyclase activity in S49 cyc<sup>c</sup> membranes was reduced approximately 65%. By contrast, cyc<sup>c</sup> complementation assays did not reveal a difference between the functional activity of G<sub>s</sub> in hearts from 30-day-old BIO 53.58 hamsters and F<sub>1</sub>B controls. Furthermore, β-adrenergic receptor stimulation of adenylyl cyclase in the membranes of the young BIO 53.58 hamsters was not significantly different from controls. The substantial alterations in G<sub>s</sub> bioactivity in hearts of the 100-day-old BIO 53.58 hamsters was not associated with alterations in the immunodetectable levels of either αG<sub>s</sub> or αG<sub>i</sub> on Western Blots. These results suggest that G protein changes are associated with ventricular dilation in BIO 53.58 hamsters and that G protein levels are not always reflective of G protein bioactivity. (Circulation 1990;81:1341–1352)

Neurohumoral signals modulate the acute changes in cardiac contractility by interacting with transmembrane signal processing systems located within the myocardial cell surface membrane. These systems receive and amplify the neurohumoral signals that regulate cardiac contractility by changes in intracellular second messengers. The primary signaling system in the heart that mediates the inotropic effects of β-adrenergic receptor agents is the receptor–G protein–adenylyl cyclase complex. This signaling system consists of three components, that is, 1) membrane receptors, 2) guanine nucleotide–binding regulatory proteins or G proteins that transduce stimulatory (G<sub>s</sub>) or inhibitory (G<sub>i</sub>) signals, and 3) the effector enzyme adenylyl cyclase that converts ATP to the intracellular second

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messenger cyclic AMP (cAMP).4,5 The G proteins are a family of highly homologous proteins that share a common heterotrimeric structure, that is, an α-subunit, a β-subunit, and a γ-subunit. At least four species of αG, having relative molecular weights of 45,000 or 52,000 have been recognized.6 These various forms are produced by alternative splicing from a single gene.6,7 In contrast, three species of αG have been identified, each of which is encoded by a distinct gene.8

In contrast to the normal heart, the failing heart is relatively insensitive to sympathetic stimuli.9 This desensitization results in part from down-regulation of β-adrenergic receptors.10 A more distal component of the receptor-adenyllyl cyclase complex, however, can also be involved, and that is the G proteins. The bioactivity of Gα1, as measured by pertussis toxin catalyzed ADP-ribosylation and adenyllyl cyclase activity is increased in the failing human heart.11 By contrast, Gα2 bioactivity is diminished in ventricular myocardiun from dogs with heart failure that results after aortic banding.12 In both situations, the net effect is an attenuation of adenyllyl cyclase activity and decreased production of the second messenger cAMP.

The BIO 14.6 strain of cardiomypathic golden Syrian hamsters is a well-studied animal model of congestive heart failure that develops the following characteristic pathological changes: cardiac myolysis at 30–40 days of age, cardiac hypertrophy at approximately 150 days of age, cardiac dilatation at approximately 250 days of age, and frank congestive failure at approximately 1 year of age.13 In previous studies of cardiac G proteins in these hamsters, we demonstrated that bioactivity of cardiac Gαs is significantly decreased before onset of pathological changes, without concomitant changes in immunochemically detectable levels of the α-subunit of Gα (αGα). This defect in αGα was muscle specific because it occurred only in cardiac and skeletal muscle.

Our finding of altered αGα bioactivity in the young BIO 14.6 cardiomyopathic hamster raised several important questions. 1) Do closely related but phenotypically distinct myopathic hamster strains demonstrate similar biochemical alterations? 2) Is progression of cardiac disease to dilation associated with abnormal Gα function? 3) Does the function of αGα correlate with responsiveness to β-adrenergic receptor agonists? To answer these questions, we assessed the activity of G proteins in hearts from BIO 53.58 Syrian hamsters, an inbred strain that arose as a spontaneous mutation of the BIO 14.6 strain (C. Van Dongen, personal communication). In contrast to the BIO 14.6 hamster, the BIO 53.58 hamsters do not develop myolysis or hypertrophy before dilation, have a significantly shorter life span, and demonstrate reduced cardiac function at an earlier age than do the BIO 14.6 hamsters.14 Therefore, the BIO 53.58 hamster provides a model of cardiac dilation that contrasts with the hypertrophic model of the BIO 14.6 strain.

Methods

Experimental Animals

Male golden Syrian hamsters (Mesocricetus auratus) were obtained from Bio Breeders (Fitchburg, Massachusetts). Two genetically defined strains were studied, the cardiomypathic BIO 53.58 hamsters and normal controls (BIO F,B). The latter strain is an F1 hybrid of strains BIO 1.5 and BIO 87.20, whereas the former strain was developed from a spontaneous mutation in the BIO 14.6 strain (C. Van Dongen, personal communication). The hamsters were allowed free access to food and water. After deep anesthesia was induced with intraperitoneal pentobarbital (60 mg/kg), hearts were excised and placed in either 1) cold 5 mM Tris-Cl (pH 7.5) containing 250 mM sucrose and 1 mM EGTA for biochemical studies, 2) cold 30 mM KCl to arrest the hearts in diastole for pathological evaluation, or 3) cold 6 mM Na-HEPES (pH 7.4) containing 140 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 16.7 mM glucose, and 2.5 mM CaCl2 for subsequent hemodynamic evaluation. These studies conformed to the guiding principles of the American Physiological Society and were approved by the animal care committee.

Cardiac Pathology

Hearts were processed for histopathological studies and quantitatively assessed for dilation as previously described.17 Briefly, after fixation by immersion in a buffered 4% formaldehyde solution for 24 hours, the hearts were sliced into 2-mm rings parallel to the atrioventricular groove from base to apex. Each slice was embedded in glycol methacrylate and cut into 3-μm-thick serial sections with an LKB Histoquant microtome (Cambridge Instruments, Deerfield, Illinois). The serial sections were cut in groups of four; one section from each group was stained with toluidine blue, a second section was stained with hematoxylin and eosin, a third section was stained with Masson’s trichrome for connective tissue, and a fourth section was stained with Von Kossa method for calcium deposits.

The image of each histological heart section was projected at a magnification of ×7.5–10.5, depending on the heart size, and traced onto paper. The cavity area of each section was then digitized, and maximal diameter was determined using a videoplan image analysis microcomputer (Carl Zeiss, Thornwood, New York). Mean cavity diameter was calculated using serial reconstruction of the histological heart rings.

Isolated Heart Perfusion

After cardiac excision, the aorta was cannulated and perfused retrograde on a modified Langendorff apparatus as previously described18 with 6 mM Na-HEPES (pH 7.4) containing 140 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 16.7 mM glucose, and 2.5 mM CaCl2. The perfuse was saturated with 100% O2 gas before use and maintained at 38°C during the experiment. Hearts were perfused using a Masterflex
Model 7018-21 constant flow pump (Cole-Parmer Instrument Co., Chicago, Illinois) with modulation of the perfusion rate in each heart to maintain a coronary perfusion pressure of 70–80 mm Hg. The flow required to maintain coronary perfusion pressure did not differ in the normal (F;3B) or myopathic (BIO 53.58) hamsters. Isovolumic developed pressure in the left ventricle was measured with a fluid-filled latex balloon inserted into the left ventricle through the mitral valve orifice. The balloon was connected with PE 190 polyethylene tubing to a Statham P23Db transducer (Gould Inc., Glen Burnie, Maryland), and pressures were continuously recorded on a Brush direct-writing recorder (Gould). Bipolar teflon-coated stainless steel electrodes were attached beneath the epicardial surface and used to pace the heart at 250 beats/min with a Grass SD-9 stimulator (Grass Instrument Co., Quincy, Massachusetts).

The hearts were perfused and allowed to equilibrate for 30 minutes to establish a stable baseline. The volume in the intraventricular balloon was then progressively increased until a left ventricular end-diastolic pressure of approximately 10 mm Hg was achieved, and the experiments were performed at this volume. DL-Isoproterenol (1 μM) was then added to the perfusion solution, and peak contractile responses were measured.  

Membrane Preparation

Cardiac membranes (particulate fractions) for adenyl cyclase and cyc− complementation assays were isolated and prepared as previously described. Briefly, ventricular myocardium was isolated and homogenized with a Polytron (Brinkmann Instruments, Westbury, New York) for less than 5 seconds in cold 5 mM Tris-Cl (pH 7.5) containing 250 mM sucrose and 1 mM EGTA. After centrifugation for 20 minutes at 1,100g (4° C), the resulting pellet was washed twice with this same solution and resuspended with a Potter-Elvejehm homogenizer. The final particulate fraction was filtered through gauze, and aliquots were stored at −70°C.

For immunochemical studies and for β-adrenergic receptor antagonist–binding studies, a cardiac membrane preparation deficient in contractile proteins was prepared. Ventricular myocardium pooled from two hearts was homogenized with a polytron in 10 mM Tris-Cl (pH 7.8)–1 mM EGTA buffer, and the resulting homogenate was added to an equal volume of 1 M KCl and stirred for 15 minutes at 4° C. After centrifugation at 49,000g for 15 minutes at 4° C, the resulting pellet was resuspended in 75 mM Tris-Cl (pH 7.4)–10 mM MgCl2 buffer and centrifuged. The final membrane pellet was resuspended in 50 mM Tris-Cl (pH 7.4) containing 250 mM sucrose and 1 mM EGTA and stored at −70°C.

Assay of Adenyl Cyclase Activity

Adenyl cyclase activity was measured as previously described. Briefly, the reaction mixture (100 μl) contained 50 mM Na-HEPES (pH 7.6), 0.5 mM MgCl2, 0.3 mM KCl, 0.1 mM [α-32P]ATP (0.1–0.2 mCi/mmol), 20 μM cAMP, 1.0 mM diithiothreitol, 5 mM creatine phosphate, 2.8 units creatine kinase, and various agonists as indicated. GTP (10 μM) was added except to reactions containing fluoride ion, forskolin, or nonhydrolyzable GTP analogues. The reaction mixtures were prepared at 4°C, and the reactions were conducted at 30°C after the addition of cardiac membranes (10 μg). The reaction was terminated at 20 minutes by adding 100 μl cold 50 mM Na-HEPES (pH 7.5) containing 2 mM ATP, 0.5 mM [3H]cAMP (0.8 Ci/mmol), and 2% sodium dodecyl sulfate (SDS) (stop buffer) and heating for 3 minutes at 90°C. cAMP was separated from ATP using Dowex-alumina chromatography. All reactions were performed in triplicate and recovery of cAMP was greater than 70%.

Cyc− Complementation Assay

The bioactivity of αG, in a particulate fraction from heart was evaluated by measuring the ability of detergent extracts to reconstitute β-adrenergic receptor stimulation of adenyl cyclase in membranes prepared from cyc− S49 murine lymphoma cells (strain 94.15.1), which are genetically deficient in αG. After propagation in Dulbecco’s modified Eagle’s medium, the cyc− cells were disrupted with a nitrogen cavitation apparatus and plasma membranes were separated using sucrose density-gradient centrifugation. The cyc− membranes were then suspended in 20 mM Na-HEPES (pH 8.0) containing 2 mM MgCl2, 1 mM EDTA, and 1 mM dithiothreitol, and stored frozen at −70°C.

The cyc− reconstitution assay was performed as previously described. In brief, cardiac membranes (particulate fraction) were incubated at 37°C for 30 minutes and then suspended in 10 mM Tris-Cl (pH 7.5) containing 0.2% (vol/vol) Lubrol-PX, 0.1 mM EDTA, 10 mM MgCl2, and 1 mM dithiothreitol for 60 minutes at room temperature. After centrifugation at 15,000g for 15 minutes (room temperature), the supernatant containing the membrane extract was removed. The cyc− complementation assay consisted of 50–60 μg of cyc− membranes, 0–20 μl of cardiac membrane extract, and reaction buffer (90 μl) containing 50 mM Na-HEPES (pH 7.4), 12.5 mM MgCl2, 0.3 mM KCl, 0.1 mM ATP, 20 μM cAMP, 0.1 mM diithiothreitol, 5 mM creatine phosphate, 2.8 units creatine kinase, 10 μM isoproterenol, 10 μM forskolin, and 10 μM Gpp(NH)p. After incubation for 20 minutes at 30°C, 10 μl of reaction buffer containing [α-32P]ATP (90 Ci/mmol) was added, and the incubation was continued for an additional 40 minutes. The reaction was stopped by adding 100 μl stop buffer and heating for 3 minutes at 90°C. cAMP accumulation was then measured as described for the adenyl cyclase assay.

Bioactivity of αG, in the membrane extracts was calculated by subtracting the endogenous activity of adenyl cyclase in the cyc− membranes from the
activity in the reconstituted membranes. Adenyl cyclase activity in the detergent extracts of the cardiac membranes was negligible. All assays were performed in triplicate. The complementation experiments reported in the present study were all performed with the same batch of nonfat membranes; therefore, data are presented as picomoles of cAMP formed per assay tube. Preliminary studies were performed with detergent extracts of cardiac membranes prepared from both BIO 53.58 and FB hamsters of both age groups to ensure that the amount of cAMP synthesized in the complementation assay was directly proportional to the amount of tissue extract added. Increasing amounts of membrane extract were used to generate a standard curve demonstrating linearity of the assay; appropriate amounts of boiled extract were added to these reactions to ensure that the volume of extract and, therefore, of detergent was equal in each reaction. The rate of cAMP synthesis remained constant during the 40-minute incubation.

**Immunochemical Determination of Levels of αG, and αG**

Polyclonal antisera specific for αG and αG were generously provided by J. Robishaw (Geisinger Clinic, Danville, Pennsylvania) and H.R. Bourne (University of California, San Francisco, California), respectively. The anti-αG antisera was directed against a synthetic peptide deduced from the nucleotide sequence at the carboxyl terminal end of bovine αG (RDHQMRHLRQYELL). The amino acid sequence at the carboxyl terminus of hamster αG, deduced from a cardiac cDNA clone is identical to this sequence (D.A. Conner, A.M. Feldman, and C. Van Dop; unpublished observations).

The relative amounts of αG in cardiac membranes from the 30-day-old and 100-day-old BIO 53.58 and FB hamsters were measured using an affinity-purified antibody from polyclonal antiserum raised against a synthetic peptide identical to a portion of the amino acid sequence of mouse αG. Cardiac membranes deficient in contractile proteins (50 μg) were suspended in electrophoresis buffer (50 μl) containing 62 mM Tris-Cl (pH 6.8), 2% (wt/vol) SDS, 10% (wt/vol) glycerol, and 0.7 M 2-mercaptoethanol, and electrophoresed on a 10% gel using SDS-polyacrylamide gel electrophoresis (PAGE). The resolved proteins were electrophoretically transferred to Immobilon PVDF membranes (Millipore Co., Bedford, Massachusetts) using 3-(cyclohexylamino)-1-propanesulfonic acid (pH 11.0) in 10% (vol/vol) methanol. The transfer membranes were then incubated for 1 hour at 37°C in 50 mM Tris-Cl (pH 8.0) containing 2 mM CaCl2, 80 mM NaCl, 5% (wt/vol) nonfat dry milk, and 0.02% (wt/vol) sodium azide (immunoblotting buffer) to which 0.2% (vol/vol) Nonidet P-40 and 10% (vol/vol) horse serum had been added. The blots were then incubated for 2 hours with antisera that had been diluted 1:1,100 in immunoblotting buffer containing 2% Nonidet P-40 and 0.2% SDS. After three washings (10 minutes) with immunoblotting buffer containing detergent, the blots were incubated with goat anti-rabbit 125I-F(ab')2 (2×106 cpm/ml) for 1 hour at room temperature. The blotting membranes were then washed twice as described above and then twice with 50 mM Tris-Cl (pH 8.0) containing 2 mM CaCl2 and 20 mM NaCl. The blots then exposed Kodak X-OMAT AR film with an intensifying screen overnight. The intensity of the autoradiographic bands was measured using either two-dimensional densitometry (Loates, Westminster, Maryland) or γ-scintigraphy of appropriate bands excised from the blot. Previous studies have demonstrated equivalent results with the two techniques. Each gel was prepared in duplicate, and three separate experiments were performed. Variations in the specific activity of the radionucleotides and exposure time of the autoradiograms resulted in varying densities of bands on autoradiographs of immunoblots from different experiments. Therefore, we standardized autoradiographic densities by including membranes from at least three control (FB) hearts in each experiment. The levels of immunochemically detectable proteins in membranes from the BIO 53.58 hearts were then calculated as a percentage of the mean of the normal controls. This method of comparing autoradiograms from different experiments has been used previously. Transfer of protein as determined by posttransfer gel staining was complete. Additionally, Ponceau S staining of the transferred blots after electrophoretic transfer demonstrated equal amounts of protein in the lanes from the FB and myopathic animals.

**Quantification of β-Adrenergic Receptors**

β-Adrenergic receptor density in membranes prepared from the BIO FB and FB 53.58 hamsters was measured using 125I-labeled cyanopindolol ([125I]ICYP)—binding as previously described. Briefly, 50 μg of cardiac membranes were incubated with varying concentrations (3–150 pM) of radiolabeled ICYP (2,200 Ci/mmol) for 2 hours at 30°C in 20 mM Tris (pH 7.5) containing 150 mM NaCl and 1 mM ascorbate. β-Adrenergic receptor-binding was assessed by measuring the amount of bound ICYP displaced by 1 μM α-t-o-propranolol. The binding parameters B and K were determined by nonlinear least-squares fitting using a computerized algorithm.

**Materials**

[α32P]ATP was obtained from ICN Biomedicals, Inc., Radiochemicals Div. (Irvine, California), [125I]ICYP and 125I-F(ab')2 fragment of rabbit antigoat IgG were from DuPont Co. (Wilmington, Delaware), culture media was from GIBCO Corp. (Grand Island, New York), Nonidet P-40 and Ponceau S were from Sigma Chemical Co. (St. Louis, Missouri), polyethylene lauryl ether (Lubrol PX) was from Pierce Chemicals Co. (Rockford, Illinois), Gpp (NH)p was from Boehringer-Mannheim (Mannheim,
FRG), and all other reagents were the highest grade commercially available.

Statistical Analysis

Differences between the means of two groups were analyzed with Student's t test.

Cardiac Pathology

At 30 days of age, cavity size was not significantly different in the hearts from the cardiomyopathic BIO 53.58 Syrian hamsters when compared with hearts from normal F1B controls. Focal regions of myocyte loss, cellular infiltration, and contraction band necrosis, however, were noted. In contrast to hearts from the 30-day-old hamsters, hearts from 100-day-old BIO 53.58 hamsters were substantially enlarged when compared with the normal controls. Mean cavity diameter of hearts from the cardiomyopathic (BIO 53.58) hamsters (4.73±0.24 mm, n=3) was significantly (p<0.02) greater than that of the F1B controls (2.82±0.45 mm). Additionally, the heart cavity area was significantly (p<0.01) greater in the hearts from the BIO 53.58 hamsters (10.29±0.77, n=3) than in the normal F1B controls (4.35±0.95). Representative sections from these hearts are shown in Figure 1. The 100-day-old hearts also had largely healed focal lesions with calcification and fibrosis. Cellular infiltration was present but was less pronounced than in the young hamsters.

Perfused Heart Studies

Comparing hearts from 3-month-old BIO 53.58 hamsters, we were unable to demonstrate a decrease in basal developed pressure when compared with nonmyopathic control hearts (Figure 2, Panel A). A previous study of 6-month-old hamsters demonstrated a small but significant decrease in baseline-developed pressure in BIO 53.58 hamsters when compared with F1B controls.15 Our failure to demonstrate a difference might have resulted from a smaller sample size, younger age of the hamsters, or use of a lower Ca2+ concentration in the perfusate. The Ca2+ concentration in the present experiments was chosen because it allowed optimal measurement of isoproterenol-stimulated contractility. A concentration of isoproterenol (1 μM) that produced submaximal inotropic effects was chosen to avoid activation of α-adrenergic receptors. Quantitative assessments were made at peak effect, and the Ca2+ concentration

**Figure 1.** Photomicrographs demonstrating representative hematoxylin and eosin-stained pathological sections from hearts of 100-day-old control (F1B) hamster (Panel A) and 100-day-old cardiomyopathic BIO 53.58 Syrian hamster (Panel B). Sections were taken at similar anatomic levels, and original magnification is ×11.

**Figure 2.** Bar graphs of left ventricular pressures in isolated perfused hamster hearts. Resting (Panel A) developed pressure and peak (Panel B) developed pressure in response to isoproterenol (1 μM) in isolated perfused hearts from 100-day-old cardiomyopathic BIO 53.58 Syrian hamsters (hatched bars) and age-matched F1B controls (open bars). Perfusions were performed as described in "Methods." Response to isoproterenol is expressed as percentage of change from baseline. Data are mean±SEM. *p<0.05.
was maintained at 2.5 mM because previous studies have demonstrated that optimal inotropic responsiveness in rodent hearts is present at low Ca²⁺ concentrations.31 Isoproterenol responsiveness as measured by the percentage of increase in developed pressure was decreased approximately 35% in the hearts from the 100-day-old BIO 53.58 hamsters when compared with hearts from age-matched controls (Figure 2, Panel B).

Adenylyl Cyclase Activity

Adenylyl cyclase activity was measured in the normal and myopathic hamsters in the presence of various pharmacological agents to assess the function of the receptor–G protein–adenylyl cyclase complex (Table 1). In particulate fractions prepared from hearts of 30-day-old BIO 53.58 hamsters, adenylyl cyclase activity measured with 10 mM GTP was the same as in comparably aged F₁B hamsters. Similarly, there was no difference in adenylyl cyclase activity between the two preparations on stimulation with either the β-adrenergic receptor agonist isoproterenol, the catalytic adenylyl cyclase activator Mn²⁺ or the nonhydrolyzable GTP analogue Gpp(NH)p. By contrast, adenylyl cyclase activity in the presence of fluoride ion was significantly less in cardiac particulate fractions prepared from the BIO 53.58 hamsters when compared with particulate fractions from F₁B controls. This disparity might be because fluoride, unlike guanine nucleotides and β-adrenergic receptor agonists, augments G₁-stimulated adenylyl cyclase activity independent of dissociation of the αβγ-heterotrimer of G₁.32 A small but significant decrease in adenylyl cyclase activity in cardiac particulate fractions prepared from BIO 53.58 hamsters was also found in the presence of forskolin, an adenylyl cyclase activator that requires αG, for optimal activation but has actions that are inhibited by guanine nucleotides.29

In the 100-day-old BIO 53.58 hamsters, which had dilated hearts, cardiac adenylyl cyclase activity was significantly reduced (Table 1). Basal adenylyl cyclase activity (with GTP) as well as activity in the presence of isoproterenol, fluoride, and forskolin was significantly reduced. These differences in adenylyl cyclase stimulation were also present when net increases were calculated. It is unlikely that an abnormality in the catalytic unit of adenylyl cyclase explained the diminished adenylyl cyclase activity in the BIO 53.58 hamsters because there was no difference between Mn²⁺-stimulated adenylyl cyclase activity in either group. Furthermore, the decrease in agonist-stimulated adenylyl cyclase activity in the 100-day-old BIO 53.58 hamsters cannot be attributed to abnormalities in myocardial catecholamine concentrations because similar results were obtained in the presence of the β-adrenergic receptor blocker propranolol (Table 2).

Basal stimulated as well as agonist-stimulated adenylyl cyclase activity in the particulate fractions prepared from 100-day-old hamsters was approximately twofold that found in membranes from the 30-day-old hamsters. This disparity might be because of age; however, because hearts from the two groups (30-day-old and 100-day-old hamsters) were processed at varying times, it is conceivable that variations in tissue processing might explain the differences.

**Cyc⁻ Complementation Assays**

The functional activity of αG, was assessed by measuring the ability of detergent extracts of cardiac particulate fractions to reconstitute β-adrenergic

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**Table 1. Adenylate Cyclase Activity in Hearts From Dilated Cardiomyopathic (BIO 53.58) Syrian Hamsters**

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>Net NaF (10 mM)</th>
<th>Net forskolin (10 μM)</th>
<th>Net iso</th>
<th>Net Gpp(NH)p (10 μM)</th>
<th>Mn²⁺ (1 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30-day-old F₁B (n=6)</td>
<td>1.7±0.05</td>
<td>2.8±0.4</td>
<td>16.8±1.0</td>
<td>6.0±0.3</td>
<td>7.4±0.6</td>
<td>6.7±0.5</td>
</tr>
<tr>
<td>BIO 53.58 (n=6)</td>
<td>1.7±0.06</td>
<td>1.1±0.1</td>
<td>13.9±0.5</td>
<td>6.2±0.4</td>
<td>7.7±0.6</td>
<td>6.7±0.7</td>
</tr>
<tr>
<td>p</td>
<td>NS</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
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</table>

| 100-day-old F₁B (n=8) | 6.9±0.4  | 11.0±0.4        | 30.1±0.8              | 9.3±0.5 | 5.6±0.5              | 6.8±0.6     |
| BIO 53.58 (n=8)       | 4.2±0.3  | 3.6±0.2         | 14.2±0.6              | 5.3±0.5 | 4.2±0.2              | 5.2±1.0     |
| p                    | <0.001  | <0.001          | <0.001                | <0.001 | <0.05                | NS          |

Values are mean±SEM. Activity is expressed as pmol cAMP/min/mg protein.

NaF, sodium fluoride; Forsk, forskolin; iso, isoproterenol; Gpp(NH)p, 5′-guanylyl imidodiphosphate.

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**Table 2. Adenylate Cyclase Activity in Hearts From Dilated (100-day-old) BIO 53.58 Syrian Hamsters in the Presence of 1 μM Propranolol**

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>Net NaF (10 mM)</th>
<th>Net forskolin (10 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F₁B (n=8)</td>
<td>1.8±0.2</td>
<td>8.6±0.6</td>
<td>29.1±1.3</td>
</tr>
<tr>
<td>BIO 53.58 (n=8)</td>
<td>1.2±0.3</td>
<td>4.7±0.5</td>
<td>15.5±1.1</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
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</table>

Values are mean±SEM. Activity is expressed as pmol cAMP/min/mg protein.
FIGURE 3. Graph of representative experiment demonstrating linear relation between amount of detergent extract from heart membranes and reconstituted adenylate cyclase activity in cyc⁻ membranes. Extracts were prepared from cardiac membranes of 30-day-old (●) and 100-day-old (○) F₁B hamsters. After reconstitution of extract with membranes prepared from cyc⁻ cells, adenylate cyclase activity was assessed in presence of 10 μM isoproterenol, 100 μM Gpp(NH)₃, and 10 μM forskolin. Points shown are mean of triplicate determinations.

receptor stimulation of adenyl cyclase in membranes from cyc⁻ S49 mouse lymphoma cells. There was a direct linear relation between the amount of cardiac extract and activation of adenyl cyclase (Figure 3). Bioactivity of α₅₇ in extracts of heart prepared from the 30-day-old BIO 53.58 and F₁B hamsters were the same. There was, however, an approximate 70% reduction of α₅₇ bioactivity in cardiac extracts from 100-day-old BIO 53.58 hamsters when compared with 100-day-old F₁B controls (Table 3). Forskolin was added to the reaction mixtures to amplify α₅₇ effects because previous studies did not demonstrate any effect on the measurement of functional α₅₇.¹¹,¹⁴ Cyc⁻ membranes genetically lack α₅₇ but contain adequate amounts of β-adrenergic receptors, α₅₁, and βγ-subunits of G protein. Therefore, the inability of detergent extract from the hearts of the 100-day-old BIO 53.58 hamsters to fully reconstitute isoproterenol-stimulated adenyl cyclase activity in the presence of forskolin and guanine nucleotides in cyc⁻ membranes suggested that there was a functional abnormality in α₅₇, preventing its stimulatory interaction with catalytic adenyl cyclase.

On a total protein basis, cardiac extracts from the older F₁B hamsters were twofold as effective in reconstituting isoproterenol-stimulated adenyl cyclase activity as extracts from the 30-day-old hamsters. This correlated with increased basal stimulated as well as agonist-stimulated adenyl cyclase activity in the older hamsters and supports the hypothesis that this change in the cardiac adenyl cyclase complex accompanies the aging process. Additionally, we have demonstrated increased immunologic α₅₇ in hearts from older hamsters when compared with younger hamsters in both groups (data not presented).

Immunochemical Quantification of G Proteins

The α₅₇ antiserum directed against the carboxyl terminal end of α₅₇ detected two peptides (Mr, 45,000 and Mr, 52,000) on immunoblots from cardiac membranes isolated from the young BIO 53.58 and F₁B hamsters (Figure 4, Panel A), and there was a linear relation between the amount of protein applied to the immunoblot and band density on the autoradiographs when 25–100 μg of protein was applied.¹⁴ Both bands could also be detected on blots prepared from membranes of 100-day-old hamsters but detection of the Mr, 45,000 peptide required longer exposure times (36–48 hours). We previously reported quantification of only the Mr, 45,000 peptide on immunoblots from crude cardiac membranes isolated from young hamsters. This disparity is because of the use of a membrane preparation deficient in contractile proteins for immunoblotting in the present study in contrast to a particulate fraction of cardiac membranes used for immunoblotting in previous studies¹⁴ (Figure 4, Panel A). As we previously reported,¹⁴ the antiserum does not cross-react with other closely related G proteins because no peptides were appreciated between Mr, 38,000 and Mr, 42,000. This is not surprising because the amino acid sequence of the carboxyl terminus of α₅₇ differs substantially from that of either α₅₁ or α₅₇. In contrast to our finding that functional activity of α₅₇ was substantially reduced in the 100-day-old BIO 53.58 hamsters, there was no difference between immunologic levels of α₅₇ in the BIO 53.58 hamsters and in the normal F₁B controls when the levels of the Mr, 52,000 peptides were quantified (Figure 5). Additionally, no difference was found between immunodetectable levels of the Mr, 45,000 peptide in the membranes prepared from the BIO 53.58 hamsters (107.8±15%, n=5) when compared with the normal controls (100.1±13%, n=5). Similarly, we were unable to appreciate differences in immunologic α₅₇ in the young BIO 53.58 hamsters and in the normal age-matched F₁B controls when either the Mr, 45,000 or Mr, 52,000 peptides were quantified (data not presented).

The relative amounts of α₅₇ in cardiac membranes from 100-day-old BIO 53.58 and F₁B hamsters were measured using an affinity-purified antibody (G12) from polyclonal antiserum raised against a synthetic peptide identical to a portion of the amino acid sequence of mouse α₅₇. In the cardiac membrane preparation used in the present study, this antibody

| Table 3. Activity of α₅₇ in Hearts From BIO 53.58 Syrian Hamsters |
|---------------------------------|-------------------|------------------|
|                                | 30-day old (n=6)  | 100-day old (n=8) |
| F₁B                            | 5.7±0.5           | 16.2±0.9         |
| BIO 53.58                      | 5.2±0.6           | 4.6±0.6          |
| p                              | NS                | <0.001           |

Activity was assessed by measuring the ability of detergent extracts to reconstitute isoproterenol-activated adenylate cyclase in membranes from cyc⁻ S49 mouse lymphoma cells, which genetically lack α₅₇. Activity is expressed as pmol cAMP formed/reaction. All assays were performed in triplicate. Values are given as mean±SEM.
detected an Mr 38,000 band that comigrated with a protein detected by the same antibody in a preparation of partially purified G protein from bovine brain as well as an Mr, 42,000 peptide (Figure 4, Panel B). There was a linear relation between the amount of protein applied to the blot (20–100 μg) and immunodetectable Mr, 38,000 and 42,000 peptides. Quantification of the relative amounts of immunodetectable αGi did not reveal a significant difference between levels of αGi in cardiac membranes from the 100-day-old BIO 53.58 and F1B hamsters (Figure 5). These results were consistent with the pharmacological studies that did not suggest an alteration in the αGi pathway.

The G12 antibody was produced using a synthetic peptide identical to a region of the deduced amino acid sequence of rat αGi-2 but differing from the homologous regions of rat αGi-1 by two amino acids and differing from αGi-3 by three amino acids. Therefore, it is conceivable that this antibody might not recognize either αGi-1 or αGi-3. Because of this possibility, we also used a second antisera raised against a synthetic peptide identical to a portion of the deduced amino acid sequence of rat αGi-1 and αGi-3 (IAlRAGML)8 to quantify immunodetectable levels of αGi. This peptide differed from the homologous peptide region on rat αGi-2 by five amino acids, two of which are Arg residue. Characterization of this antisera demonstrated its ability to detect αGi-1 and αGi-3 but not αGi-2 in membrane preparations of human heart (unpublished observations). This antisera detected a single band with an approximate molecular weight of 42,000 in membrane preparations of hamster heart. Furthermore, no differences were found between relative levels of this protein in hearts from the BIO 53.58 hamsters and the normal F1B controls. Although the amino acid sequences of the rat αGiS might not be identical to those of the homologous hamster αGiS, there are probably not large differences because the entire amino acid sequence of hamster αGi, (D.A. Conner, A.M. Feldman, C. Van Dop; unpublished observations) is identical to that of rat αGi.8

Quantification of β-Adrenergic Receptors

Similar to results from our previous studies14 and studies by others54 in the BIO 14.6 hamsters, there was no difference between either β-adrenergic receptor antagonist affinity or receptor density in cardiac

**FIGURE 4.** Immunoblots of cardiac membrane proteins resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred from gels to support membranes and incubated sequentially with affinity-purified antibodies selective for either αGi, (Panel A) or αGi, (Panel B) and then with 125I-labeled goat anti-rabbit IgG F(ab')2 fragments as described in “Methods.” Each gel lane contained 50 μg cardiac membrane protein. Panel A: Lane 1, 100-day-old BIO 53.58 hamster; lane 2, 100-day-old F1B hamster; lane 3, 100-day-old F1B hamster (approximately 48-hour exposure); lane 4, 30-day-old F1B hamster (overnight exposure); and lane 5, 100-day-old F1B hamster, particulate preparation (lanes 3 and 5 were immunoblotted simultaneously. Panel B: Lane 1, 100-day-old F1B hamster with 3 μg partially purified G protein from bovine brain added; lane 2, 100-day-old BIO 53.58 hamster. All immunoblots exposed film overnight except as noted.
membranes prepared from 100-day-old BIO 53.58 hamsters when compared with the F1B controls (Table 4). These results support the hypothesis that diminished receptor–adenylyl cyclase coupling in these cardiac preparations results from altered bioactivity of G,

### Discussion

The cardiomyopathic hamster strain BIO 14.6 was identified in 1969,35 and subsequent inheritance patterns of the trait indicated that the propensity for developing cardiomyopathy was inherited as an autosomal recessive trait. The cardiomyopathic hamster strain (BIO 53.58) used for these studies was discovered as a “spontaneous mutation” among inbred BIO 14.6 hamsters (C. Van Dongen, personal communication). The original BIO 53.58 hamsters have been outcrossed once into BIO 1.5 strain. The propensity for developing cardiomyopathy behaves as a single locus autosomal recessive trait in both cardiomyopathic strains (C. Van Dongen, personal communication). Therefore, the causative mutation is probably in the same locus. Although hamsters of both these cardiomyopathic strains ultimately develop terminal dilated congestive heart failure, their phenotypes differ because BIO 14.6 hamsters develop cardiac hypertrophy before dilation, whereas BIO 53.58 hamsters do not.16 Despite these gross phenotypic differences, both strains develop myocardial intracellular calcium deposits before dilation,36 with a more severe lesion in BIO 53.58 hamsters than in comparably aged BIO 14.6 hamsters (H.P. Weisman, unpublished observations).

Although both strains of the cardiomyopathic Syrian hamster have been studied as models of congestive heart failure, the etiologic genetic defect that causes heart failure in these hamsters is still unknown. A wide variety of biochemical abnormalities have been described in dilated hearts from older members of both myopathic hamster strains.37–39 Only a few cardiac abnormalities, however, occur in animals before cardiac dilation, and they include abnormal calcium uptake,40 increased calcium antagonist receptors,17 and decreased G, bioactivity.14 The guanine nucleotide–binding protein G, has recently been shown to directly activate voltage-gated calcium channels as well as adenylyl cyclase.41,42 Therefore, our previous demonstration in young BIO 14.6 hamsters showing that bioactivity of cardiac G, is deficient in stimulating adenylyl cyclase,14 coupled with others’ observations that cardiac calcium uptake was excessive in response to β-adrenergic receptor stimulation,40 had led us to hypothesize that a biochemical defect in αG, differentially affects its ability to stimulate adenylyl cyclase and gated calcium channels. The different results of the present study in closely related BIO 53.58 hamsters, however, suggest there is a role for other proteins or enzymes that control G, signal transduction.

In contrast to our previous results in the cardiomyopathic BIO 14.6 hamsters, isoproterenol activation of adenylyl cyclase in heart membranes from young BIO 53.58 hamsters was not different from that in F1B controls. Furthermore, cardiac extracts from the BIO 53.58 hamsters normally reconstituted isoproterenol-stimulated adenylyl cyclase activity of S49 cye- membranes, whereas extracts from BIO 14.6 hamsters of similar age had noticeably reduced activity.14 Similar to the BIO 14.6 hamsters, however, young cardiomyopathic Syrian hamsters of the BIO 53.58 strain demonstrated decreased adenylyl cyclase activity in the presence of the G protein effectors fluoride ion43 or forskolin, each of which require αG, for maximal stimulation of catalytic adenylyl cyclase.44 It is possible that the selective loss of fluoride and forskolin stimulation of adenylyl cyclase in the myopathic BIO 53.58 hamsters despite an apparently normal β-adrenergic receptor–adenylyl cyclase coupling is because of a subtle specific defect in αG, that is manifested when G, is in its heterotrimeric form but not when it is dissociated in the presence of agonist occupancy of the β-adrenergic receptor. Considerable evidence supports the hypothesis that fluoride can activate adenylyl cyclase in cell membranes without dissociation of the G, heterotrimer.32.33 Fluoride stimulates adenylyl cyclase activity in frog erythrocytes, S49 lymphoma cells, and human platelets without affecting high-affinity binding of β-adrenergic receptor agonists.45 By contrast, the further addition of guanine nucleotides to these fluoride-treated cells decreases affinity of β-adrenergic receptors for agonist.45 Additionally, fluoride can mask the effects of the G,-inhibition on adenylyl cyclase as fluoride-stimulated adenylyl cyclase activation is unaffected by pretreatment with pertussis toxin under appropriate conditions.46 If fluoride-stimulated adenylyl cyclase activity reflected a balance of the activation of both G, and G, we would have expected that blockade of the inhibitory effects of G, with pertussis toxin would allow unopposed activation of G, and, therefore, increased adenylyl cyclase activity. G, also enhances stimulation of adenylyl cyclase by forskolin by a mechanism that is independent of G protein dissociation. In human heart membranes, forskolin stimulates adenylyl cyclase maximally in the absence of guanine nucleotides with decreased effect after addition of either GTP or Gpp(NH)pp.29

Although the young BIO 53.58 hamster demonstrated only mild alterations in receptor–adenylyl cyclase coupling, progression of cardiac disease in
these hamsters to dilated congestive failure was associated with pronounced diminution in the functional activity of $G_i$. Basal adenyl cyclase activity, as well as that stimulated by isoproterenol, forskolin, and fluoride, was significantly decreased in cardiac membranes from 100-day-old BIO 53.58 hamsters. Additionally, the 65% decrease in $\alpha G_i$ bioactivity found in the cardiac membranes from the BIO 53.58 hamsters by using the S49 ccc" complementation assays suggests that the hearts of the older BIO 53.58 hamsters have a functional abnormality of $\alpha G_i$, that is similar to that in the young BIO 14.6 hamsters. Because the ccc" membrane contains an excess of free $\beta y$-subunits, $\alpha G_i$, and adrenergic receptors, it is unlikely that an alteration in a component of the receptor--$G$ protein--adenylyl cyclase complex other than $\alpha G_i$ could explain our results. Neither altered myocardial catecholamine levels, decreased $\beta$-adrenergic receptor density, nor changed $\beta$-adrenergic receptor affinity are likely to be the cause of the discrepancy in agonist-stimulated adenyl cyclase between the BIO 53.58 and F1B hamsters because 1) neither the $\beta$-adrenergic receptor density nor antagonist affinity were altered in dilated hearts from the BIO 53.58 hamsters, and 2) addition of propranolol, a $\beta$-adrenergic receptor antagonist, did not eliminate the difference in basal stimulated or G protein-agonist--stimulated adenyl cyclase activities. The decreased $\alpha G_i$ activity in the cardiac membranes of the BIO 53.58 hamsters was not associated with altered levels of immunoreactive $\alpha G_i$, implying a qualitative rather than quantitative defect. These $G$ protein alterations differ from those reported for the failing human heart11 but are similar to alterations found in cardiac $G_i$ protein activity after aortic banding in dogs12; however, those investigators did not quantify $G_i$ using immunologic methods. It is, therefore, not clear whether cardiac $G_i$ bioactivity was altered in this dog model.

Our finding that there is a progressive abnormality in receptor--adenyl cyclase coupling that alters $\alpha G_i$ bioactivity during development of cardiac dilation in the absence of alterations in immunochromically detectable levels of $\alpha G_i$ suggests that a protein or enzyme system occurs that covalently modulates $\alpha G_i$ protein function in the heart. It is well known that $G$ proteins can be posttranslationally modified by ADP-ribo sylation47,48 and phosphorylation.49,50 Such alterations could be important in moment-to-moment modulation of $G$ protein activity. $G$ proteins synthesized in bacteria by recombinant DNA techniques42,51,52 also lack posttranslational modification; however, these proteins have a more far reaching defect as they demonstrate a permanent unalterably low specific activity. $G$ proteins can also undergo myristoylation,53 a reaction that can be extremely important for proper targeting of the proteins to the membranes. Alternative splicing of $\alpha G_i$ gene transcripts, which yields mRNA encoding four isoforms of $\alpha G_i$ in human brain,6,54 however, might explain our findings. The relative amounts of $\alpha G_i$ in the M1, 45,000 and M2, 52,000 forms are the same in hearts from the BIO 53.58 and F1B hamsters; however, two of the alternative splicings described by Bray et al6 result in insertion or deletion of a serine residue in either the M1, 45,000 or M2, 52,000 isofoms of $\alpha G_i$. The location of these serine residues is distant from the carboxyl terminus, where the antibodies used in our studies bind. These Ser residues might be phosphorylation sites (as previously described for $\alpha G_i$49,50) for protein kinases regulating $G_i$ function. Further characterization of this system that modulates $G_i$ bioactivity is beyond the scope of this investigation; however, the myopathic hamster might provide a model system in which to study posttranslational modification of $G$ proteins. This information might provide insights regarding the regulation of cardiac responsiveness to extracellular signals in the failing heart.

We present evidence that $G$ protein abnormalities in the myopathic Syrian hamster are strain specific, occurring before the onset of pathological changes in the hypertrophic BIO 14.6 hamster while occurring commensurately with ventricular dilation in the BIO 53.58 myopathic hamster strain. The significance of these studies lies in this disparity between the results of the present study and those previously reported in the BIO 14.6 hamster. Because the $\alpha G_i$ defect in the BIO 53.58 myopathic hamster is not present early in life but appears after the onset of heart failure, the defect is probably not causally related to the onset of the cardiomyopathy but rather is the consequence of hereditary cardiomyopathy. The substantial decrease in $G_i$ bioactivity accompanying dilation in the 100-day-old BIO 53.58 hamsters, however, can contribute to both diminished $\beta$-adrenergic receptor--adenyl cyclase coupling and to decreased hemodynamic responsiveness to $\beta$-adrenergic receptor stimulation in the hearts of these hamsters. Furthermore, in contrast to our earlier study in young BIO 14.6 hamsters, the results of the present study suggest that deficient $G_i$ function results from an altered biochemical pathway that posttranslationally modifies $\alpha G_i$. Finally, the present study confirms previous suggestions that immunochromically detectable levels of $G_i$ might not reflect $G_i$ bioactivity.55 Therefore, this study points out the necessity of performing both qualitative and quantitative assessments of $G$ protein activity to correctly interpret the role of $G$ proteins in cardiac disease.

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