INCREASED LEVELS OF INHIBITORY G PROTEIN IN MYOCARDIUM WITH HEART FAILURE

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To identify any differences in inhibitory G protein (Gi) attributable to species or the cause of heart failure, we studied the changes in this protein in different animal models of heart failure: 1) different species; rats vs. hamsters (F1B) with cardiomyopathy induced by adriamycin (ADR) and 2) different etiologies; rats with ischemic heart failure (IHD) due to coronary artery ligation vs. rats with cardiomyopathy induced by ADR and F1B (20-week-old) hamsters with cardiomyopathy induced by ADR vs Syrian hamsters BIO 14.6 (40-week-old) with genetic cardiomyopathy, using Western blotting methods and ADP-ribosylation. We also sought to determine whether changes in the amount of Gi protein reflected the regulation of adenylate cyclase. The amount of immunodetectable Gi rose by 35% (p<0.05) in ADR rats, 25% (p<0.05) in ADR hamsters, 15% (p<0.05) in IHD rats, and 28% (p<0.05) in BIO 14.6 hamsters, as compared with control rats, F1B (20-week-old) hamsters, sham-operated control rats, and F1B (40-week-old) hamsters, respectively. Assessment of Gi by pertussis toxin-catalyzed ADP-ribosylation revealed increases in Gi of 24% (p<0.05) in ADR rats and of 44% (p<0.05) in BIO 14.6 hamsters, as compared with their respective controls. Gi function, as assayed by the acetylcholine-induced inhibition of adenylate cyclase, also increased. Thus, Gi protein appears to contribute to the changes in signal transduction in myocardium with heart failure.

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The receptor-G protein-adenylate cyclase complex in the myocardial cell membrane is an important pathway of sympathetic nerve stimulation that regulates cardiac function. In the failing heart, sympathetic neural function is substantially altered. Heart failure is associated with an increase in systemic plasma norepinephrine levels, although the failing heart exhibits decreased responsiveness to sympathetic nerve stimulation! This decreased responsiveness results in part from down-regulation of β-adrenergic receptors. However, recent studies have demonstrated substantial changes in G-protein function in failing human and animal hearts. The function of stimulatory G protein (Gs) decreases in heart failure. In contrast, the function of inhibitory G protein (Gi) is generally believed to increase in heart failure. However, there have been several reports of either no change or actual decreases in Gi function in failing hearts. These discrepancies may reflect differences in the etiologies of left ventricular failure in the model systems, in the species of animals studied in the state of left ventricular com-

Key words:
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ADR-induced cardiomyopathic rats and hamsters
Cardiomyopathic BIO 14.6 hamsters

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pensation, or in the methods of measurement (ADP-ribosylation vs Western blotting). Therefore, to investigate the possible contribution of the species of animal and/or the etiology of heart failure, we studied the changes in this protein in different animal models of heart failure: 1) different species; rats vs. hamsters (F1B) with cardiomyopathy induced by Adriamycin (ADR) and 2) different etiologies; rats with ischemic heart failure (IHD) due to coronary artery ligation vs rats with cardiomyopathy induced by ADR and F1B (20-week-old) hamsters with cardiomyopathy induced by ADR vs Syrian hamsters BIO 14.6 (40-week-old) with genetic cardiomyopathy. We also quantitated Gi by Western blotting using an anti-Gi antibody and by ADP-riboseylation. In addition, we sought to determine whether changes in the amount of Gi reflected those in adenylate cyclase (AC) activity.

**METHODS**

**Experimental Animals**

We studied four models of heart failure: 1) different species; rats vs hamsters (F1B) with cardiomyopathy induced by Adriamycin (ADR) and 2) different etiologies; rats with ischemic heart failure (IHD rats) due to coronary artery ligation vs rats with cardiomyopathy induced by ADR and F1B (20-week-old) hamsters with cardiomyopathy induced by ADR vs Syrian hamsters BIO 14.6 (40-week-old) with genetic cardiomyopathy.

Six-week-old Wistar rats were treated with Adriamycin (ADR rats, n=15) and age-matched Wistar rats served as controls (control rats, n=15). Sixteen-week-old F1B hamsters were treated with Adriamycin (ADR F1B, n=10) and age-matched F1B hamsters served as controls (F1B-20w, n=10). One month after a single intraperitoneal (i.p.) administration of ADR 5 mg/kg light microscopy of heart tissue revealed diffuse degenerative and necrotic changes. Myocardial infarction was produced in 10-week-old male Wistar rats weighing 260-290 g by the method of Morita et al. After performing a left thoracotomy under anesthesia, the proximal left coronary artery was ligated. A sham operation was performed in an identical manner, but without ligation of the coronary artery, in control rats. Of the 19 rats in the group with ischemic heart failure, 4 died within 4 weeks of the operation. Four weeks after the operation, the hearts were removed. When the hearts of all of the animals were excised, we checked to determine whether any of the areas of the free wall showed thinning. Five rats were excluded from the study because the infarcted area was only small or moderate. The remaining 10 rats were used for Western blotting and measurement of AC activity. The same number of sham-operated rats served as controls in each group. Since the ventricular free wall of the infarct zone showed thinning associated with fibrosis and was not suitable for this study, we used the ventricular septum of the non-infarct zone. The BIO 14.6 Syrian hamster (BIO 14.6 hamster) is a well-studied animal model of congestive heart failure. Forty-week-old male BIO 14.6 Syrian hamsters (n=15) were used, with age-matched F1B hamsters serving as controls (F1B-40w, n=15). BIO 14.6 and F1B hamsters were obtained from Biobreeders (Fitchburg, MA, USA).

They received humane care and were maintained in accordance with Rules for Animal Experimentation of the Hyogo College of Medicine.

**Cardiac Myocyte Membranes**

Cardiac ventricular myocytes were isolated from each species by the methods of Powell and Twist with some modifications. HEPES perfusion buffer (1.2 mM MgSO_4_, 5 mM KCl, 5 mM NaHCO_3_, 10 mM glucose, 20 mM HEPES and 120 mM NaCl, pH 7.4) was gassed at room temperature with 95% O_2_, 5% CO_2_. After the animals had been anesthetized with ether, the hearts were removed immediately and subjected to retrograde perfusion with the above buffer in a by Langendorff's method. Once the hearts had been rinsed free of blood, the perfusion medium was replaced with HEPES buffer containing collagenase type 1, 0.06 mg/ml (Sigma Chemical Co., St. Louis, Mo, USA) and bovine serum albumin (BSA), 1 mg/ml. This enzymatic perfusion medium was recirculated at 37 °C for 20 min. The atria and attached connective tissue were trimmed away and the ventricles were placed in 20 mM HEPES buffer containing BSA,
1 mg/ml, divided into 30 pieces, and incubated at 37 °C for 10 min with constant shaking and gassing. The resulting cell suspensions were filtered through gauze and centrifuged for 3 min at 50×g. Cell pellets were homogenized in a polytron (Type PT20, Kinematica, Switzerland) in a hypotonic medium consisting of 10 mM triethanolamine (TEA)-HCl buffer (pH 7.4) and 5 mM EDTA, and the homogenates were centrifuged at 10,000×g for 5 min. The pellets, washed twice with 10 mM TEA-HCl buffer, were used as the membrane fraction of cardiac myocytes.

Measurement of Gi-Protein by Western Blotting

Myocardium was homogenized for 30 sec in a 10-fold excess of ice-cold buffer containing 25 mM Tris-HCl, 1 mM DTT, 1 mM EGTA, 1 mM MgCl₂, 1 mM APMSF (pH 7.4) by using a polytron at setting 7. The homogenate was centrifuged at 100,000×g for 60 min. The pellet, washed twice in incubation buffer, yielded a crude fraction of myocardial membrane. Western blotting techniques were modified according to Towbin et al.¹⁷ Polyclonal antiserum was raised in rabbits (Dupont/NEN, Boston, MA, USA) against the αi1 and αi2 subunits of Gi, and reacted strongly against the respective subunits and weakly against αi3 and αo. SDS-PAGE was performed according to the method of Laemmli using a 12% polyacrylamide gel. The proteins (50 μg for Gi α) on the gel were transferred electrothermally to nitrocellulose sheets for 12 h at 8 V/cm, as described by Towbin et al.¹⁷ The sheets were incubated with TBS buffer (50 mM Tris-HCl at pH 7.5 and 0.2 M NaCl) containing 0.05% Tween-20 and 5% BSA for 2 h at room temperature. They were then incubated for 1 h at room temperature with a 1/1000 dilution of anti-Gs α or a 1/500 dilution of anti-Gi α polyclonal antibody in TBS buffer containing 5% BSA. The sheets were then washed 4 times for 15 min each (60 min total) at room temperature with TBS buffer containing 0.05% Tween 20. To detect antibody attached to the sheets, they were incubated for 1 h at room temperature with ¹²⁵I-labelled anti-rabbit Ig (specific activity 2.5 μCi/ml, Amersham, Boston, MA, USA). Thereafter, the sheets were washed eight times for 15 min each with TBS containing 0.05% Tween-20, dried, and autoradiographed at −80 °C (Kodak X film with intensifying screens). Molecular sizes were determined by including prestained molecular weight marker proteins. The amounts of Gi α in the 40-kDa bands were estimated by a gamma counter (5360, Packard Instrument. Co., Meriden, CT, USA). The amount of Gi α (cpm/μg) was defined as the difference between the 40-kDa bands and the background.

[³²P]ADP-Ribosylation of G-Proteins by Pertussis Toxin (PTX)

The reaction medium for ADP-ribosylation consisted of 50 mM TEA-HCl buffer (pH 7.4), 1 mM EDTA, 1 mM DTT, 1 mM ATP, 2 mM MgCl₂, 1 μM [³²P] NAD, PTX, 10 mM thymidine and cardiac myocyte membranes (10 μg/100 μl) or purified Gi protein (0.4 μg/100 μl). Gi proteins were partially purified from rat brain by the method of Katada et al.¹⁸ The reaction was carried out at 25 °C for 90 min and was stopped by adding 60% trichloroacetic acid (TCA). The mixture was then centrifuged at 10,000×g for 5 min. The pellet was washed three times with 10 mM TEA-HCl buffer at 4 °C and then heated in Laemmli sample buffer at 90 °C for 3 min and subjected to electrophoresis on a linear gradient gel.¹⁹ After autoradiography, the area of absorbance of the 40–41kDa band was measured with a chromatograph (Shimazu, CS-930, Kyoto, Japan).²⁰

Radioligand Binding Assay

We measured β-adrenergic receptor density in cardiac membrane using [¹²⁵I]-iodocyanopindolol (ICYP) (specific activity 2200 Ci/mmol, Dupont/NEN Research Products, Boston, MA, USA) binding. Myocardium was homogenized for 30 sec in a 10-fold excess of ice-cold buffer containing 5 mM Tris-HCl, 0.25 M sucrose, and 1 mM MgCl₂ (pH 7.4) in a polytron at setting 7. The crude homogenate was centrifuged at 400×g for 10 min, and the supernatant was centrifuged at 10,000×g for 10 min. The resultant supernatant was centrifuged at 100,000×g for 30 min, and the pellet was washed twice in incubation buffer (50 mM Tris-HCl, 10 mM MgCl₂ [pH 7.4]), to yield a crude fraction of
TABLE I BODY WEIGHT, HEART WEIGHT, AND THE RATIO OF HEART WEIGHT TO BODY WEIGHT IN CONTROL RATS, ADR RATS, SHAM-OPERATED RATS, IHD RATS, FIB-20w, ADR FIB, FIB-40w AND BIO 14.6 HAMSTERS

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Body Wt (g)</th>
<th>Heart Wt (mg)</th>
<th>Heart Wt/Body Wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control rats</td>
<td>15</td>
<td>288±16</td>
<td>865±30</td>
<td>3.0±0.1</td>
</tr>
<tr>
<td>ADR rats</td>
<td>5</td>
<td>234±16*</td>
<td>708±18*</td>
<td>3.1±0.1</td>
</tr>
<tr>
<td>FIB-20w</td>
<td>10</td>
<td>110±11</td>
<td>418±20</td>
<td>3.8±0.2</td>
</tr>
<tr>
<td>ADR-FIB</td>
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<td>102±4</td>
<td>378±23</td>
<td>3.7±0.3</td>
</tr>
<tr>
<td>Sham-ope rats</td>
<td>10</td>
<td>289±14</td>
<td>896±31</td>
<td>3.1±0.2</td>
</tr>
<tr>
<td>IHD rats</td>
<td>10</td>
<td>131±10</td>
<td>891±45</td>
<td>3.3±0.4</td>
</tr>
<tr>
<td>FIB-40w</td>
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<td>128±3</td>
<td>490±50</td>
<td>3.9±0.3</td>
</tr>
<tr>
<td>BIO 14.6</td>
<td>15</td>
<td>128±3</td>
<td>510±30</td>
<td>4.0±0.1</td>
</tr>
</tbody>
</table>

Values are mean±SEM. *p<0.05 vs control rats. Wt: weight

myocardial membrane. This cardiac membrane was incubated with various concentrations (100–400 pM) of $[^{125}I]$ICYP at 25°C for 20 min. The samples were then filtered through Whatman GF/F filters (Whatman, Clifton, NJ, USA), and the filters were washed three times with 5 ml of ice-cold incubation buffer. The radioactivity retained on the filters was counted with a gamma counter (B460C, Packard Instrument Co., Downers Grove, IL, USA). Specific binding was defined as the difference between total binding and binding in the presence of 10 pM propranolol.

We also measured muscarinic receptors in ADR rats compared to that in control rats. ADR rat cardiac myocytes, suspended in HEPES buffer, were incubated with various concentrations (0.2–4 nM) of $[^3H]$quinuclidinyl benzilate (QNB) (specific activity 33.1 Ci/mmol, NEN, Boston, MA, USA) at 37°C for 30 min. Specific binding was defined as the difference between total binding and binding in the presence of 10 pM atropine.

Adenylate Cyclase Assay
The method of Salomon et al.\textsuperscript{21} was used to measure basal adenylate cyclase activity as well as that in the presence of 20 mM sodium fluoride (NaF) or 10 μM acetylcholine (ACh) with 20 mM sodium fluoride. We also measured cyclic-AMP levels after stimulation with forskolin (10 μM). The assay buffer contained 50 μM \( [\alpha^32P] \) ATP, 2 mM MgCl\(_2\), 0.1 mM EDTA, 1 mM 3-isobutyl-1-methylxanthine, 5 mM creatine phosphate, 0.4 mg/ml creatine kinase, 0.1 mM cyclic AMP, 2 mg/ml BSA, 10 mM cholic acid and the additions indicated in 50 mM trisethanolamine/HCl (pH 7.4), in a total volume of 100 μl. Homogenates (15 μg of protein per tube) were preincubated with the reaction mixture in the absence or presence of NaF, forskolin or ACh with NaF for 4 min at 30°C. Thereafter, labelled ATP was added and the adenylate cyclase reaction was continued for 10 min at 30°C. To evaluate the function of Gi, we calculated percent changes in cyclic-AMP levels in response to ACh after NaF stimulation, expressed as % inhibition by

\[
ACh = \frac{(NaF-ACh)/NaF} \times 100.
\]

Protein Concentration
Protein concentration was determined by the method of Lowry et al.\textsuperscript{22} using bovine serum albumin standards.

Statistical Evaluation
Results are expressed as the mean±SEM. Statistical significance was estimated with Student's t test for unpaired observations. A p value of less than 0.05 was considered statistically significant.

RESULTS
Cardiac Pathology and Clinical Signs
No ADR rats or hamsters died during the study. The morphologic characteristics of the heart failure models are shown in Table I. The bodies and hearts of the ADR rats

_N. Circulation J. Vol. 58, December 1994_
weighed significantly less than those of the controls. There were no significant differences in the heart/body weight ratio between ADR and control rats. There were no significant differences in body weight, heart weight, or the ratio of heart/body weight between F1B ADR, IHD rats, and BIO14.6 and their respective controls. One month after a single i.p. administration of ADR, 5 mg/kg, examination of the myocardium of rats by light microscopy revealed diffuse degenerative and necrotic changes, including numerous vacuolizations (Fig. 1B). Fig. 1A shows the myocardium of a control rat. The hearts of the 40-week-old male BIO 14.6 hamsters were dilated; light microscopy revealed calcification and fibrosis of the myocardium (Fig. 1D). Fig. 1C shows the myocardium of an F1B hamster (F1B-40w) as a control. The hearts of ADR F1B hamsters showed the same changes as ADR rats (data not shown). The ventricular septum of IHD rats showed a compensatory hypertrophy. All of the models with heart failure showed clinical signs of congestive heart failure, i.e., tachypnea, bristling of the hair, and pulmonary congestion. Pleural effusion or ascites was observed in 7 ADR rats (32%), 2 ADR hamsters (20%), and 3 IHD rats (30%). All of the BIO 14.6 hamsters exhibited edema and the color of their eyes had changed to black.

G-protein Quantification by Western Blotting

Fig. 2 shows Western blotting of Gi α, using a polyclonal antiserum against α 1 and α 2, on a blot of ventricular myocardial membrane from a control rat, ADR rat,
Sham-operated rat, IHD rat, F1B-20w, ADR F1B, F1B-40w and BIO 14.6 hamster in a representative experiment. The right lane (Fig. 2) shows the results obtained with 0.6 μg of partially purified Giα from rat brain. As shown in Table II, the amount of immunodetectable Gi rose by 35% (p<0.05) in ADR rats, 25% (p<0.05) in ADR hamsters, 15% (p<0.05) in IHD rats, and 28% (p<0.05) in BIO 14.6 hamsters, as compared with those in control rats, F1B-20w hamsters, sham-operated rats, and F1B-40w hamsters, respectively.

TABLE II IMMUNODETECTABLE α Gi ON WESTERN BLOTS IN HEARTS OF CONTROL RATS, ADR RATS, F1B-20w, ADR F1B HAMSTERS, SHAM-OP DE RATS, IHD RATS, F1B-40w AND BIO 14.6 HAMSTERS

<table>
<thead>
<tr>
<th></th>
<th>Western Blots radioactivity (cpm/μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control rats</td>
<td>5</td>
</tr>
<tr>
<td>ADR rats</td>
<td>5</td>
</tr>
<tr>
<td>F1B-20w</td>
<td>5</td>
</tr>
<tr>
<td>ADR F1B</td>
<td>5</td>
</tr>
<tr>
<td>Sham-ope rats</td>
<td>5</td>
</tr>
<tr>
<td>IHD rats</td>
<td>5</td>
</tr>
<tr>
<td>F1B-40w</td>
<td>5</td>
</tr>
<tr>
<td>BIO 14.6</td>
<td>5</td>
</tr>
</tbody>
</table>

Values are mean±SEM. *p<0.05 vs. their controls, respectively.

[32P] ADP-Ribosylation of G-proteins by PTX

ADP-ribosylation of the 40-41 kDa proteins by PTX in rat or hamster cardiac myocyte membranes was linear for about 60 min, and was almost saturated after 90 min. Addition of more PTX or [32P]NAD to the reaction mixture after 90 min did not increase the amount of ADP-ribosylation by PTX. There was no signal when myocardial membrane was absent (Fig. 3, lane1). When only myocardial membrane was present, there was a band at 50 kDa (Fig. 3, lane2). The membrane fraction yielded a band at 40-41 kDa when both myocardial membrane and PTX were present (Fig. 3, lane 3). The intensity of these bands was significantly reduced by ADP-ribosylation in the presence of 100 μM GTPγS (guanosine 5'-O-[3-triophosphoribonucleotide]) (Fig. 3, lane 4). Partially purified Gi protein from rat brain (41 kDa) was also ADP-ribosylated by PTX (Fig. 3, lane 5). This finding indicates that the 40-41 kDa band in the cardiac myocyte membrane fraction included Gi protein. Fig. 4 shows ADP-ribosylation of G protein by PTX (band at 40-41 kDa) in myocardial membrane from a control rat, ADR rat, F1B-40w and BIO 14.6 hamster in a representative.

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Gi Protein in Cardiomyopathy

Fig. 3. Representative autoradiogram of ADP-ribosylation of rat heart ventricular cell membranes by PTX. The membrane fraction yielded a band at 40-41 kDa, the intensity of which was significantly reduced by ADP-ribosylation in the presence of 100 μM GTP γS. Purified Gi protein (41 kDa) was also ADP-ribosylated by PTX (lane 5). Molecular weight standards are shown in kDa on the left. PTX: pertussis toxin. Lane 1: in the absence of myocardial membrane fraction. Lane 2: in the presence of only myocardial membrane. Lane 3: in the presence of both myocardial membrane and PTX. Lane 4: in the presence of membrane, PTX and GTP γS.

Fig. 4. Representative autoradiogram of ADP-ribosylation in control, ADR rats, F1B and BIO 14.6 hamsters. (F1B and BIO 14.6: 40-week-old hamster)

Experiment. Other spots in each lane indicate myocardial membrane and PTX, as shown in Fig. 3. The ADP-ribosylation assay of the 40-41 kDa protein showed linear results over a range of 5-70 μg of membrane protein (r=0.99). However non-membrane proteins, as found in mitochondria and the sarcoplasmic reticulum, may also be included when total protein is measured by Lowry's method. G protein exists on the cytosolic surface of the plasma membrane. 5'-Nucleotidase frequently behaves as a plasma-membrane enzyme in subcellular fractionations of mammalian tissues. 5'-Nucleotidase activity by the method of Newby et al was linear over a range of 5-70 μg of membrane protein as measured by Lowry's method (r=0.97). Thus, the amount of protein measured by Lowry's method is a reliable index of Gi protein concentration. As shown in Table III, absorbance of the 40-41 kDa band (Gi protein) was significantly increased by 24% in ADR rats, as compared to that in the controls (p<0.05). Absorbance of this band was also significantly increased by 44% in BIO 14.6 hamsters, as compared to that in F1B hamsters (p<0.05).

β-Adrenoceptor and Muscarinic Receptor Assay

The number of β-adrenoceptors (Bmax) was significantly decreased by 61.8% in ADR rats and by 71.9% in BIO14.6, as compared with control rats and F1B hamsters, respectively, with no changes in Kd (Table IV).

Muscarinic receptors were measured using [3H] QNB as a radioligand. There were no differences in Bmax or Kd between ADR and control rats (Bmax; ADR: 179±28 fmol/mg protein vs controls: 183±24 fmol/mg protein, Kd; ADR: 0.76±0.08 nM vs controls: 0.66±0.03 nM.)
TABLE III  ADP-RIBOSYLATION BY PTX IN HEARTS OF CONTROL, ADR RATS, FIB AND BIO 14.6 HAMSTERS

<table>
<thead>
<tr>
<th></th>
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<th>ADP-ribosylation Area of absorbance (densitometric units)</th>
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</thead>
<tbody>
<tr>
<td>control rats</td>
<td>5</td>
<td>1001 ± 66</td>
</tr>
<tr>
<td>ADR rats</td>
<td>5</td>
<td>1243 ± 69*</td>
</tr>
<tr>
<td>FIB-40w</td>
<td>5</td>
<td>738 ± 100</td>
</tr>
<tr>
<td>BIO 14.6</td>
<td>5</td>
<td>1063 ± 92*</td>
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</table>

Values are mean ± SEM. *p<0.05 vs their controls, respectively.

TABLE IV  β-ADRENOCEPTORS IN HEARTS OF CONTROL, ADR RATS, FIB-40W AND BIO 14.6 HAMSTERS

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Bmax (fmol/mg protein)</th>
<th>Kd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control rats</td>
<td>5</td>
<td>61.8 ± 4.5</td>
<td>300 ± 170</td>
</tr>
<tr>
<td>ADR rats</td>
<td>5</td>
<td>38.2 ± 14.5*</td>
<td>350 ± 170</td>
</tr>
<tr>
<td>FIB-40W</td>
<td>5</td>
<td>72.0 ± 11.7</td>
<td>41 ± 11</td>
</tr>
<tr>
<td>BIO 14.6</td>
<td>5</td>
<td>51.8 ± 8.6*</td>
<td>37 ± 13</td>
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</table>

Values are mean ± SEM. *p<0.05 vs their respective controls.

TABLE V  ADENYLATE CYCLASE ACTIVITY IN MEMBRANES PREPARED FROM HEARTS OF CONTROL RATS, ADR RATS, FIB-20w, ADR FIB HAMSTERS, SHAM-OPE RATS, IHD RATS, FIB-40w AND BIO 14.6 HAMSTERS

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<th></th>
<th>n</th>
<th>Basal</th>
<th>NaF</th>
<th>Forskolin</th>
<th>NaF-ACh</th>
<th>% inhibition by ACh</th>
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<tbody>
<tr>
<td>Control rats</td>
<td>5</td>
<td>10.5 ± 0.5</td>
<td>74.5 ± 1.5</td>
<td>76.1 ± 2.1</td>
<td>17.5 ± 0.5</td>
<td>89.0 ± 2.5</td>
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<tr>
<td>ADR rats</td>
<td>5</td>
<td>9.5 ± 0.8</td>
<td>70.5 ± 0.4</td>
<td>72.6 ± 1.3</td>
<td>10.5 ± 0.5*</td>
<td>98.3 ± 0.6*</td>
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<tr>
<td>FIB-20w</td>
<td>5</td>
<td>6.0 ± 0.2</td>
<td>37.4 ± 1.2</td>
<td>36.6 ± 0.4</td>
<td>9.8 ± 0.4</td>
<td>87.9 ± 2.5</td>
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<td>ADR FIB</td>
<td>5</td>
<td>4.8 ± 0.2</td>
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<td>34.2 ± 1.2</td>
<td>5.8 ± 0.6*</td>
<td>96.7 ± 2.9*</td>
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<tr>
<td>Sham-ope rats</td>
<td>5</td>
<td>10.6 ± 0.4</td>
<td>65.4 ± 0.5</td>
<td>59.1 ± 0.8</td>
<td>16.1 ± 0.5</td>
<td>90.0 ± 1.1</td>
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<td>IHD rats</td>
<td>5</td>
<td>11.1 ± 1.0</td>
<td>50.1 ± 1.1*</td>
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<td>6.2 ± 0.4</td>
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<td>5.0 ± 0.4</td>
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<td>27.2 ± 1.8*</td>
<td>5.4 ± 0.6*</td>
<td>98.0 ± 0.5*</td>
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Values are mean ± SEM. *p<0.05 vs their controls, respectively.
IHD rats: rats with ischemic failing heart, Sham-ope rats: rats with sham operation.

Adenylate Cyclase Activity
AC activities in each model under various conditions are summarized in Table V. Although the basal and NaF- and forskolin-stimulated activities of AC were similar in ADR rats and hamsters compared with their respective controls, ADR rats and hamsters showed significantly lower cyclic-AMP levels in response to ACh after NaF stimulation than their respective controls. The percentage change in cyclic-AMP levels after ACh was significantly (p<0.05) larger in ADR animals than in their controls. NaF- and forskolin-stimulated AC activities were also significantly smaller in IHD rats and BIO 14.6 hamsters than in their control sham-operated rats and FIB-40w hamsters, respectively. The percentage change in the cyclic-AMP level after ACh in IHD rats and BIO14.6 was significantly greater (p<0.05) than in their respective controls (Table V).

DISCUSSION
We investigated the changes in Gi proteins in four animal models of heart failure with different etiologies in different species, using two different techniques: i.e., ADP-ribosylation and Western blotting. G protein is thought to be a heterotrimer composed of three distinct subunits: α, β, and γ. Gi protein is ADP-ribosylated by PTX. ADP-ribosylation reflects neither the absolute amount nor the function of Gi where there is a low concentration of substrates or in the presence of materials that attenuate the ribosylation response. The amount of Gi can
be determined quantitatively using Western blotting with an anti-Gi protein antibody. Although it has been reported that the determined amount may not completely reflect the level of functional G protein, we used Western blotting as an alternative method to ADP-ribosylation to determine the amount of Gi. Rats and hamsters with ADR-induced cardiomyopathy, IHD rats and BIO 14.6 hamsters showed an increase in Gi proteins based on Western blotting. These increases were consistent with findings using ADP-ribosylation in ADR rats and BIO 14.6 hamsters. Sen et al. reported that Gi was increased 1.6 times in BIO 14.6 hamsters at the age of 32 weeks, as assayed by ADP-ribosylation. Feldman et al. reported that in humans with heart failure due to dilated cardiomyopathy (DCM), the activity of a 40,000-mol wt PTX substrate (Gi α) was increased by 36%; these authors suggested Gi α as a new marker for failing myocardium that may account, at least in part, for the reduction in contractility of cardiac muscle. Böhmer et al. also demonstrated an increase in Gi α in human hearts with DCM, as assayed by ADP-ribosylation. Our findings were consistent with these results, and suggested that no interspecies difference exists in this respect between the rat, hamster, and human. Measurement methods do not affect these results, since similar increases in Gi were seen with both ADP-ribosylation and Western blotting. In contrast, it has been reported that the amount of Gi, as measured by both ADP-ribosylation and Western blotting, was unchanged in BIO 14.6 hamsters at 29 days of age. These results differ from ours, and may be explained by differences in the age of the hamsters or in the stage of cardiac failure. Our results showed that the amount of Gi increased in hearts with adriamycin-induced cardiomyopathy, ischemic cardiomyopathy or genetic cardiomyopathy. As indicated above, Gi is increased in patients with heart failure due to DCM. However, Gi has been reported to be decreased or unchanged in patients with heart failure due to ischemic heart disease. Human ischemic cardiomyopathic hearts may include ischemic myocardium. In contrast, we used the ventricular septum of the non-infarcted zone, which showed compensatory hypertrophy. Furthermore, the amount of Gi has been shown to differ between BIO 14.6 and BIO 53.58 hamsters, which do not develop the cardiac hypertrophic phase preceding cardiac failure. Therefore, heart failure with a different etiology may influence the amount of Gi.

In this study, an increase in Gi was found in hearts with cardiac failure, as compared with that in their controls. To investigate whether this increase plays an increased inhibitory role in failing hearts, we examined the degree of inhibition of AC activity in response to ACh after stimulation with Gs. It is well known that the number of β-adrenoceptors is decreased in failing hearts due to down-regulation. Since isoproterenol stimulates AC activity through β-adrenoceptors, we used NaF, which stimulates Gs directly. The cyclic-AMP levels in response to ACh after stimulation with NaF were significantly lower in ADR rats, ADR F1B, IHD rats and BIO14.6, as compared with the levels in their respective controls. These findings suggest that the increase in the amount of Gi may have functional relevance. Muscarinic receptors, which exert anti-adrenergic effects on the heart, couple to AC via a PTX-sensitive Gi α. Böhmer et al. reported that the amount of Gi and Gi function in human heart failure due to DCM were both increased, independently of A1-adenosine or muscarinic receptors, which is consistent with our results. However, Chidiac et al. reported that although the number of muscarinic receptors in spontaneously cardiomyopathic Syrian hamsters (TO strain) did not differ from those in random-bred (RB) controls, the TO strain showed impaired muscarinic signalling, perhaps due to an altered relationship between Gi and the effector, the receptor, or both. Moreover, the number of muscarinic receptors and their affinity were decreased in dogs with heart failure due to chronic pressure overload, even though Gi α was unchanged. Although we measured the number of muscarinic receptors in ADR rats in our study, we did not measure this value in BIO14.6 hamsters. The change of in cyclic-AMP levels in response to ACh after NaF stimulation was increased in BIO14.6. These findings suggest that the function of the muscarinic-Gi system is increased in BIO14.6.

Stimulation of β-receptors is known to in-
crease AC activity through Gs. A decrease in the number of \( \beta \)-receptors has been reported in human heart failure. These decreases in \( \beta \)-receptors are thought to be due to down-regulation. However, reports of changes in the number of \( \beta \)-receptors in human and animal heart failure models conflict, in that this value has been reported to increase, remain unchanged or decrease. In this study, although the number of \( \beta \) receptors was decreased in ADR cardiomyopathy, there was no difference in AC activity after NaF or forskolin stimulation, as compared with that in controls. These results suggest that there is no change in the function of Gs in ADR cardiomyopathy, which is consistent with the results of Fu et al, who detected no alterations in Gs mRNA in ADR rats. In contrast, a decrease in Gs function has been reported in a reconstitution model using S49 murine mouse lymphoma cells in BIO 14.6 hamsters at 29 days of age! In our study, NaF and forskolin stimulation resulted in decreased AC activity in IHD rats and BIO 14.6 hamsters. These results suggest that the amount and/or function of Gs in IHD rats and BIO 14.6 hamsters is decreased. Quantitation of changes in Gs in IHD rats and BIO 14.6 by Western blotting will help to clarify this point in further studies. Although Gs function was decreased, the percentage changes in cyclic-AMP levels after ACh stimulation were significantly greater in IHD rats and BIO 14.6 than in their respective controls. These results suggested that the function of Gi in IHD rats and BIO 14.6 is increased, and that this increase might reflect an increase in the amount of Gi. The amount and/or function of Gi was increased in ADR rats and hamsters, IHD rats and BIO 14.6 hamsters. In contrast, the function of Gs was decreased in IHD rats and BIO 14.6.

In our model of ADR cardiomyopathy, we administered ADR to rats and hamsters only at a dose of 5 mg/kg i.p., and studied the heart after 4 weeks. Light microscopy showed changes characteristic of ADR cardiomyopathy. Olson et al reported vacuolar degeneration of myocardial cells in animal models of heart failure, and Lefrak et al found the same vacuolar degeneration at autopsy in humans. Our ADR model in rats and hamsters also showed vacuolar degeneration in myocardial cells, which is one of the characteristics of myocardial injury by ADR. Furthermore, pleural effusion or ascites was shown in 32% (7/22) of the rats and 30% (3/10) of the hamsters. The number of \( \beta \) receptors in ADR rats was significantly decreased, as compared with that in controls. Therefore, administration of ADR at 5 mg/kg to rats serves as a reasonable model of heart failure.

Although the role of Gi in the regulation of AC activity in failing hearts has not yet been elucidated, our results suggest that down-regulation of \( \beta \)-receptors and accentuated Gi function induce a decrease in AC activity and cyclic-AMP production. Decreased AC activity and cyclic-AMP production may play an important role either in protecting myocytes from the increased sympathetic tone in heart failure or in accelerating the deterioration of cardiac function. Our study suggests that the increased amount and function of Gi in the failing heart accounts for the reduction in AC activity and cyclic-AMP production. A recent study has shown that increased \( \beta \)-adrenoceptor stimulation induced an increase in myocardial Gi \( \alpha \)-mRNA without affecting Gs \( \alpha \)-mRNA. These results suggest that increased sympathetic activity may induce a \( \beta \)-adrenoceptor-mediated increase in myocardial Gi in heart failure.

In conclusion, there were no differences in Gi attributable to the different species of animals or to the different etiologies of heart failure. Assessment of Gi by immunochemical quantification using antisera revealed increased Gi in ADR rats, ADR hamsters, IHD rats and BIO 14.6 hamsters, as compared with the values in their respective controls. The function of Gi, as assayed by the ACh-stimulated activity of AC, was also increased. Thus, Gi appears to be involved in the changes in signal transduction in myocardium with heart failure.

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