



generously provided by Dr. R. R. Reed (Johns Hopkins University School of Medicine, Baltimore, MD).

**Animals.** Three genetically distinct strains of male golden Syrian hamsters (*Mesocricetus auratus*) were obtained from Bio Breeders (Fitchburg, MA): (a) cardiomyopathic animals (BIO 14.6), (b) animals without propensity for cardiomyopathy ( $F_1$  hybrid of strains BIO 1.5 and BIO 87.20, designated  $F_1B$ ), and (c)  $F_1$  hybrids of BIO 14.6 and BIO 87.20 (designated  $F_1M$ ). The animals were fed *ad libitum* on a diet containing adequate trace elements, vitamin E, thiamine, and other nutrients for which deficiency is known to cause cardiomyopathy in animals (14). After deep anesthesia was induced with intraperitoneal pentobarbital (60 mg/kg), venous blood was collected and organs were excised. Hearts were placed in cold buffer: 20 mM Tris-Cl (pH 7.5), 150 mM NaCl. Brain, liver, and hind limb muscle were rapidly frozen in liquid nitrogen and stored at  $-70^\circ\text{C}$  before preparation of membranes.

**Preparation of membranes.** Cardiac ventricular tissue was isolated, stripped of adipose tissue, weighed, and minced with scissors. Tissue from the hearts of two hamsters was homogenized (Polytron, Brinkmann Instruments Co., Inc., Westbury, NY) for  $< 5$  s in 5 mM Tris-Cl (pH 7.5), 250 mM sucrose, 1 mM EGTA buffer, and centrifuged for 20 min at 1,100 g ( $4^\circ\text{C}$ ). The resulting pellet was washed twice and resuspended with a Potter-Elvehjem homogenizer. The final particulate fraction was filtered through gauze, aliquoted, and stored at  $-70^\circ\text{C}$ . Storage for up to 6 mo had no effect on adenylate cyclase or G protein activity. Tissue from hind limb muscles were prepared in a similar manner. Proteins were measured (15) using bovine serum albumin as standard. Membranes deficient in contractile proteins were prepared for receptor antagonist binding experiments (16). Tissue was disrupted with a Polytron in 10 mM Tris-Cl buffer (pH 7.8) containing 1 mM EGTA. The homogenate was added to an equal volume of 1 M KCl, stirred for 15 min in the cold, and centrifuged at 49,000 g for 15 min ( $4^\circ\text{C}$ ). The resulting pellet was resuspended in 75 mM Tris-Cl buffer (pH 7.5) with 10 mM  $\text{MgCl}_2$  and centrifuged again. The final pellet was resuspended in buffer containing 50 mM Tris-Cl (pH 7.4), 250 mM sucrose, 1 mM EGTA buffer, and stored at  $-70^\circ\text{C}$ .

Erythrocyte membranes were prepared from human or hamster blood after lysing cells in 5 mM  $\text{Na-PO}_4$  (pH 8) (17). Membranes were prepared from whole brain as described by Worley et al. (18). Liver was homogenized in 1 mM  $\text{NaHCO}_3$ , filtered through gauze, and centrifuged at 1,500 g for 10 min ( $4^\circ\text{C}$ ). The pellet was washed twice and resuspended in buffer containing 5 mM Tris-Cl (pH 7.5), 250 mM sucrose, 1 mM EGTA, 1 mM  $\text{MgCl}_2$ , and 1 mM DTT.

**Assay of adenylate cyclase.** Adenylate cyclase was measured as described (19). In brief, cardiac membranes were incubated in a reaction mixture (100  $\mu\text{l}$ ) containing 50 mM Na-Hepes (pH 7.6), 0.5 mM  $\text{MgCl}_2$ , 0.3 mM KCl, 0.1 mM  $[\alpha\text{-}^{32}\text{P}]\text{ATP}$  (0.1–0.2 mCi/mmol), 20  $\mu\text{M}$  cAMP, 1.0 mM DTT, 1.0  $\mu\text{M}$  DL-propranolol, 5 mM creatine phosphate, 2.8 U of creatine kinase, and agonists indicated in the figure legends and tables. GTP (10  $\mu\text{M}$ ) was added to basal and isoproterenol-stimulated reactions. The reaction mixture was prepared at  $4^\circ\text{C}$  and the reaction was carried out at  $30^\circ\text{C}$ . After a 20-min incubation period, the reaction was terminated by adding 100  $\mu\text{l}$  of buffer (stop buffer) containing 50 mM Na-Hepes (pH 7.5), 2 mM ATP, 0.5 mM  $[\text{}^3\text{H}]\text{cAMP}$  (0.8 Ci/mol), and 2% (wt/vol) SDS and heating for 3 min at  $90^\circ\text{C}$ . cAMP was fractionated using Dowex-alumina chromatography (20). Recovery was 70–90% and all reactions were performed in triplicate with  $< 10\%$  variation.

**Cyc $^-$  complementation assay.** The capability of detergent extract from cardiac membranes to reconstitute  $\beta$ -adrenergic stimulation of adenylate cyclase in membranes of the  $G_s$ -deficient cyc $^-$  S49 murine lymphoma cell was measured using a modification of a previously described technique (5). Cyc $^-$  S49 lymphoma cells (strain 94.15.1) were obtained from the University of California Cell Culture Facility (San Francisco, CA) and propagated in Dulbecco's modified Eagle's medium containing 25 mM Na-Hepes (pH 7.4) and 10% (vol/vol) heat-inactivated horse serum (21). Plasma membranes were prepared as described (22) utilizing a nitrogen cavitation apparatus (Parr Instru-

ment Co., Moline, IL) to disrupt the cells and sucrose density-gradient separation to purify the membranes. The cyc $^-$  membranes were suspended at a concentration of  $\approx 3$  mg/ml in buffer containing 20 mM Na-Hepes (pH 8.0), 2 mM  $\text{MgCl}_2$ , 1 mM EDTA, and 1 mM DTT, frozen, and then stored at  $-70^\circ\text{C}$ .

Cardiac membranes were incubated at  $37^\circ\text{C}$  for 20 min and then centrifuged at 15,000 g for 5 min at room temperature. The pellet was resuspended in buffer containing 10 mM Tris-Cl (pH 7.5), 0.2% (vol/vol) Lubrol-PX, 0.1 mM EDTA, 10 mM  $\text{MgCl}_2$ , and 1 mM DTT and agitated for 60 min at room temperature. The incubate was centrifuged at 15,000 g for 15 min at room temperature, and the supernatant (membrane extract) was removed and utilized for assay of G protein activity. Cyc $^-$  membranes (50–60  $\mu\text{g}$ ) and cardiac membrane extract (0–20  $\mu\text{l}$ ) were incubated in reaction buffer (90  $\mu\text{l}$ ) containing 50 mM Na-Hepes (pH 7.4), 12.5 mM  $\text{MgCl}_2$ , 0.3 mM KCl, 0.1 mM ATP, 20  $\mu\text{M}$  cAMP, 0.1 mM DTT, 5 mM creatine phosphate, 2.8 U of creatine kinase, 10  $\mu\text{M}$  isoproterenol, and either 10  $\mu\text{M}$  Gpp(NH)p or 10  $\mu\text{M}$  GTP $\gamma\text{S}$  for 20 min ( $30^\circ\text{C}$ ). Forskolin (10  $\mu\text{M}$ ) was also included in some experiments as noted. Reaction buffer (10  $\mu\text{l}$ ) containing  $[\alpha\text{-}^{32}\text{P}]\text{ATP}$  (90 Ci/mmol) was then added and the incubation was continued for 40 min. The reaction was stopped by adding 100  $\mu\text{l}$  of stop buffer and heating to  $80^\circ\text{C}$ ; and cAMP accumulation was measured as described for the adenylate cyclase assay. Adenylate cyclase activity in the detergent extracts from membrane extracts was negligible; however, the activity and endogenous activity of the cyc $^-$  membranes were subtracted to calculate the increase in adenylate cyclase activity resulting from addition of  $\alpha G_s$ . Preliminary studies using extracts from each tissue indicated that the amount of cAMP synthesized was directly proportional to the amount of tissue extract added and that the rate of cAMP synthesis remained constant during the 40-min incubation. In order to maintain comparable concentrations of protein and detergent in these preliminary experiments, the reaction mixtures were supplemented with membrane extracts that had been heated to  $90^\circ\text{C}$  for 10 min to inactivate  $G_s$  protein. As all complementation studies were completed using a single batch of cyc $^-$  membranes, the experimental results are expressed as picomoles cAMP produced  $\pm$  SEM.

**Bacterial toxin catalyzed ADP-ribosylations.** Cardiac membranes (75  $\mu\text{g}$ ) were centrifuged at 15,000 g and resuspended in 100  $\mu\text{l}$  of a buffer containing 50 mM Tris-Cl (pH 8.0), 10  $\mu\text{M}$   $[\alpha\text{-}^{32}\text{P}]\text{NAD}$  (20 Ci/mmol), 6 mM  $\text{MgCl}_2$ , 10 mM thymidine, 2 mM GTP, 2.5 mM ATP, 40 mM isoniazid, and 50  $\mu\text{g}/\text{ml}$  pertussis toxin. Before use, pertussis toxin was activated by incubation with 100 mM DTT. The reaction mixture was incubated for 90 min at  $30^\circ\text{C}$ . After centrifugation (15,000 g for 5 min at room temperature), the membranes were washed with buffer containing 50 mM Tris-Cl (pH 8.0), 6 mM  $\text{MgCl}_2$ , 1 mM EDTA, 146 mM sucrose before suspension in electrophoresis buffer (50  $\mu\text{l}$ ) containing 62 mM Tris-Cl (pH 6.8), 2% (wt/vol) SDS, 10% (wt/vol) glycerol, 0.7 M 2-mercaptoethanol, and electrophoresed on a 7.5% gel using SDS-PAGE (23). The gel was stained with Coomassie Blue, dried on cellulose, and exposed X-OMAT AR film (Eastman Kodak Co., Rochester, NY) for  $\approx 38$  h at  $-70^\circ\text{C}$  with an intensifying screen (Quanta III-T, DuPont Co.). The relative autoradiographic intensities for radiolabeled pertussis toxin substrates were determined using two-dimensional densitometry (Loates, Inc., Westminster, MD). The relative intensity of the 40,000-mol wt band on autoradiographs varied in different experiments as a result of variations in the film exposure time and the specific activity of the radiolabeled substrate. Therefore, we standardized autoradiographic densities obtained from separate experiments by including membranes from at least four normal hamster hearts in each experiment and expressing the levels of ADP-ribosylated proteins in membranes from cardiomyopathic hearts as a percentage of the mean of the normal controls. This method of quantifying G protein levels in cardiac tissue has been used previously (5).

G protein substrates of cholera toxin were assayed in an analogous manner as described (5). Membranes ( $\approx 100$   $\mu\text{g}$ ) were resuspended in 100  $\mu\text{l}$  of buffer containing 100 mM K- $\text{PO}_4$  (pH 7.0), 2 mM GTP, 2.5

mM ATP, 20 mM thymidine, 20 mM arginine, 10 U aprotinin, and 10  $\mu$ M [ $^{32}$ P]NAD (20 Ci/mmol). The cholera toxin was preactivated by incubating in 10 mM DTT for 30 min at 30°C.

**Immunoblotting.** Cardiac membranes (50  $\mu$ g for  $\alpha$ G<sub>s</sub>; 40  $\mu$ g for  $\alpha$ G<sub>i</sub>) were centrifuged at 15,000 *g* for 4 min, resuspended in electrophoresis buffer, resolved on a 10% gel using SDS-PAGE, and electrophoretically transferred using 3-(cyclohexylamino)-1-propanesulfonic acid (pH 11.0) in 10% (vol/vol) methanol to Immobilon PVDF membranes for immunoblotting. After transfer, the membranes were incubated for 1 h at room temperature in immunoblotting buffer (50 mM Tris-Cl (pH 8.0), 2 mM CaCl<sub>2</sub>, 80 mM NaCl, 5% (wt/vol) nonfat dry milk, and 0.02% (wt/vol) sodium azide) to which 0.2% (vol/vol) Nonidet P-40 and 10% (vol/vol) horse serum had been added. The blots were then incubated with immunoblotting buffer containing antiserum (diluted 1:1,000), 2% Nonidet P-40, and 0.2% SDS for 2 h before being washed three times for 15 min with immunoblotting buffer containing 2% Nonidet P-40 and 0.2% SDS. The antigen-antibody complexes were detected by then incubating the blots with goat anti-rabbit  $^{125}$ I-F(ab')<sub>2</sub> ( $2 \times 10^6$  cpm/ml) for 1 h at room temperature. The blots were again washed as described above and washed twice (15 min) in immunoblotting buffer containing 0.2% (vol/vol) Nonidet P-40. The blots then exposed Kodak X-OMAT AR film overnight with an intensifying screen. The intensity of autoradiographic bands was measured using two-dimensional densitometry and comparisons were performed as described for analysis of ADP-ribosylations. In addition  $^{125}$ I-F(ab')<sub>2</sub> binding was quantified by excising appropriate bands from Western blots and measuring  $^{125}$ I using a gamma-counter (TM Analytic Inc., Elk Grove Village, IL). The two methods gave comparable results.

Preliminary experiments demonstrated complete peptide transfer from the polyacrylamide gel. Additionally, Ponceau Red staining of the PVDF membrane demonstrated comparable protein concentrations in the normal and myopathic groups. To determine that the proteins were completely bound to the PVDF membrane under our transfer conditions, a second layer of the PVDF support membrane was placed next to the primary membrane during the transfer. This second membrane showed no protein binding by Ponceau Red staining.

**Northern blot and dot blot analysis of RNA.** Total RNA was purified from hearts, and blots were prepared as described (24). cDNA (1.8 kb) encoding the  $\alpha$ -subunit of rat G<sub>s</sub> was radiolabeled (10<sup>9</sup> cpm/ $\mu$ g DNA) with [ $\alpha$ - $^{32}$ P]dCTP using a random priming method (25). After prehybridization, the blots were hybridized with the radiolabeled cDNA probe (10<sup>6</sup> cpm/ml) for 48 h at 42°C before being washed and exposing Kodak X-OMAT AR film with an intensifying screen. Autoradiographic densities were quantified using two-dimensional densitometry.

**Quantitation of  $\beta$ -adrenergic receptors.**  $\beta$ -Adrenergic receptor density in cardiac membranes was measured using  $^{125}$ I-ICYP binding as previously described (26). Cardiac membranes ( $\approx 50$   $\mu$ g) were incubated for 2 h at 30°C with varied concentrations (3–150 pM) of  $^{125}$ I-ICYP (2,200 Ci/mmol). Specific  $\beta$ -adrenergic receptor binding was defined as bound ICYP displaced by 1  $\mu$ M DL-propranolol, and the binding parameters  $B_{\max}$  and  $K_d$  were determined by nonlinear least squares fitting utilizing a previously defined computerized algorithm (27).

**Statistics.** Differences between two groups were analyzed with Student's *t* test. For comparison of three groups, a one-way analysis of variance was employed using the Bonferroni correction with  $\alpha = 0.05$ . Values presented are means  $\pm$  SEM (28).

## Results

**Experimental animals.** The weights of hearts from 29-d-old animals were the same in the normal animals (F<sub>1</sub>B) (286  $\pm$  13 mg, *n* = 10), the BIO 14.6 hamsters (289  $\pm$  13 mg), and the F<sub>1</sub> hybrid animals (289  $\pm$  9 mg). In addition, microscopic exami-

Table I. Adenylate Cyclase Activity in Membranes Prepared from Hearts of Normal and Cardiomyopathic Syrian Hamsters

Agonists	F <sub>1</sub> B	F <sub>1</sub> M	BIO 14.6	P
pmol cAMP/mg protein per min				
GTP (10 $\mu$ M)	1.1 $\pm$ 0.1	1.2 $\pm$ 0.1	1.3 $\pm$ 0.1	NS
Mn <sup>2+</sup> (10 mM)	2.1 $\pm$ 0.2	2.1 $\pm$ 0.2	2.8 $\pm$ 0.2	NS
Fluoride (10 mM)	5.4 $\pm$ 0.4	4.9 $\pm$ 0.2	3.8 $\pm$ 0.2	<0.025
Forskolin (10 $\mu$ M)	20.1 $\pm$ 1.1	22.7 $\pm$ 1.7	16.1 $\pm$ 0.9	<0.025
Isoproterenol (100 $\mu$ M) + GTP	3.2 $\pm$ 0.3	3.4 $\pm$ 0.2	2.2 $\pm$ 0.5	<0.01

Adenylate cyclase activity was assayed in membranes prepared from normal (F<sub>1</sub>B) animals, animals heterozygous for the cardiomyopathic trait (F<sub>1</sub>M), and cardiomyopathic (BIO 14.6) animals in the presence of the indicated agonists. Values represent the mean  $\pm$  SE (*n* = 5) and all assays were performed in triplicate. Activities in the presence of isoproterenol, fluoride, and forskolin are the net stimulation over basal (10  $\mu$ M GTP). The effects of Mn<sup>2+</sup> were assayed in the absence of Mg<sup>2+</sup>.

nation of ventricular sections stained with hematoxylin and eosin revealed no pathologic abnormalities, particularly myolysis or myocyte necrosis.

**Adenylate cyclase activity.** Differences in activation of cardiac adenylate cyclase from the three different groups of 29-d-old hamsters by various pharmacologic agents suggested that the BIO 14.6 hamster has an abnormality in cardiac signal transduction via G<sub>s</sub>. Basal adenylate cyclase activity with GTP was not significantly different in membranes prepared from ventricles of the BIO 14.6, normal, or F<sub>1</sub>M animals (Table I). Similarly, adenylate cyclase activity in the presence of 10 mM manganous ion, a direct activator of catalytic adenylate cyclase was not different. The latter finding suggests that the amount of catalytic adenylate cyclase was the same in membrane preparations from all animals. By contrast, adenylate cyclase activity in the presence of either fluoride, forskolin, or isoproterenol was significantly less in cardiac membranes from the BIO 14.6 hamsters than in cardiac membranes from either the normal or F<sub>1</sub> hybrid animals (Table I). Fluoride stimulates adenylate cyclase by activating G<sub>s</sub> (29). Forskolin requires  $\alpha$ G<sub>s</sub> and guanine nucleotides to activate adenylate cyclase (30). The diminished effect of isoproterenol probably resulted from altered G<sub>s</sub> function, as the number and the affinity of cardiac  $\beta$ -adrenergic receptors (for ICYP) (Table II) were unaltered in the BIO 14.6 hamsters. These results suggest that the hearts from BIO 14.6 animals had altered coupling of G protein effects to catalytic adenylate cyclase. It is not surprising that

Table II.  $\beta$ -Adrenergic Receptors in Hearts of 29-d-old Normal and Cardiomyopathic Syrian Hamsters

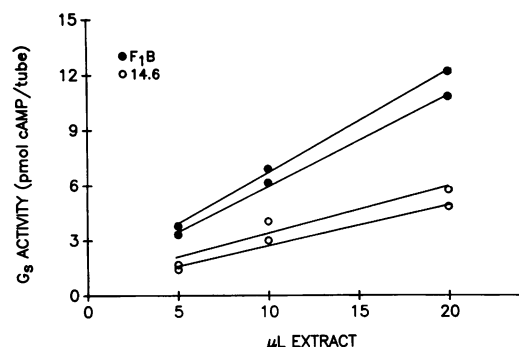
Hamsters	Dissociation constant	Maximal ICYP binding
	pM	fmol/mg protein
Normal (F <sub>1</sub> B)	43 $\pm$ 12	113 $\pm$ 4
Cardiomyopathic (BIO 14.6)	35 $\pm$ 7	100 $\pm$ 9

Values are mean  $\pm$  SE (*n* = 4).

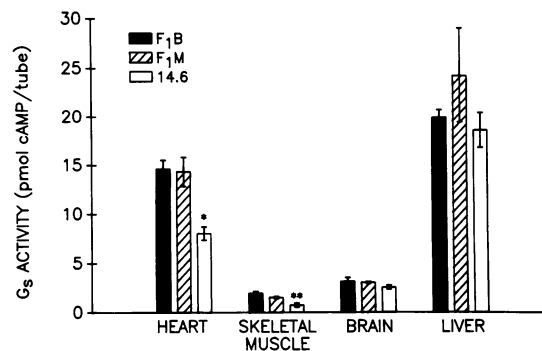
adenylate cyclase activity under basal (GTP) conditions in the BIO 14.6 animals was unchanged as similar results have been reported in renal cortex from patients with pseudohypoparathyroidism who have a generalized genetic defect in  $\alpha G_s$  (31). Preliminary studies of cardiac adenylate cyclase in another cardiomyopathic hamster strain (100-d BIO 53.58) yielded similar changes in adenylate cyclase activity (Feldman, A. M., unpublished observations). To investigate further the altered coupling of  $\beta$ -adrenergic receptors to catalytic adenylate cyclase, we quantified both  $G_s$  and  $G_i$  proteins.

**Quantitation of  $G_s$  activity by  $cyc^-$  complementation.** The amount of functional  $\alpha G_s$  activity in hamster tissues was assessed by measuring the ability of detergent extracts of membrane preparations to recouple  $\beta$ -adrenergic receptors and adenylate cyclase in membranes from  $cyc^-$  S49 murine lymphoma cells. Under the conditions of our assay, there was a direct, linear relation between the amount of cardiac membrane extract added and activation of adenylate cyclase (Fig. 1). This linear relation was found with cardiac extracts from all three groups of hamsters, as well as with extracts from other hamster tissues (data not shown).

Bioactivity of  $\alpha G_s$  in cardiac membranes from BIO 14.6 hamsters was  $\approx 50\%$  less ( $1.1 \pm 0.1$  pmol cAMP per assay tube,  $n = 5$ ,  $P < 0.01$ ) than in normal F<sub>1</sub>B animals ( $2.3 \pm 0.05$ ) when measured in the presence of isoproterenol and the nonhydrolyzable GTP analogue Gpp(NH)p. Similarly, in the presence of isoproterenol and the nonhydrolyzable GTP analogue GTP $\gamma$ s, extracts from BIO 14.6 animals were only 50% ( $P < 0.01$ ) as effective in reconstituting adenylate cyclase activity in  $cyc^-$  membranes. In some experiments, forskolin was added to the reaction mixture to amplify the activity of the reconstituted system (5). This addition had no effect on the measurement of functional  $\alpha G_s$  as an identical  $\approx 50\%$  decrease in the activity of  $\alpha G_s$  in the BIO 14.6 hamster was appreciated (Fig. 2). A comparable reduction of  $\alpha G_s$  activity was found in skeletal muscle of the BIO 14.6 animals, whereas  $\alpha G_s$  activity in membranes prepared from either pooled erythrocytes (data not shown), brain, or liver was the same in the three groups. A



**Figure 1.** Linear relation between the amount of detergent extract from heart membranes and reconstituted adenylate cyclase activity in  $cyc^-$  S49 lymphoma cell membranes. Extracts were prepared from cardiac membranes of (●) normal F<sub>1</sub>B or (○) cardiomyopathic BIO 14.6 hamsters and mixed with  $cyc^-$  membranes. Adenylate cyclase activity was assessed in the presence of 10  $\mu$ M isoproterenol, 100  $\mu$ M Gpp(NH)p, and 10  $\mu$ M forskolin. Points represent the mean of triplicate determinations. Results of two representative experiments are shown.

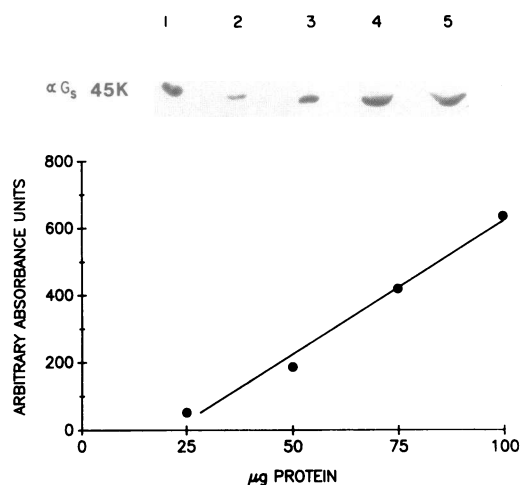


**Figure 2.** Activity of  $\alpha G_s$  in various tissues from normal (F<sub>1</sub>B), heterozygous (F<sub>1</sub>M), and cardiomyopathic (14.6) Syrian hamsters as measured by  $cyc^-$  complementation assay. Assay reactions contained 10  $\mu$ M isoproterenol, 100  $\mu$ M Gpp(NH)p, and 10  $\mu$ M forskolin. Results are mean  $\pm$  SEM ( $n = 5$ ). \* $P < 0.001$ , \*\* $P < 0.01$ .

mixed extract containing equal amounts of cardiac extract from the control ( $2.2 \pm 0.4$  pmol cAMP per tube,  $n = 5$ ) and BIO 14.6 ( $1.3 \pm 0.4$ ) had activity approximately midway ( $1.6 \pm 0.3$ ) between the activities of either pure extract, suggesting that an inhibitor of adenylate cyclase activation was not present in the BIO 14.6 cardiac membranes. The  $cyc^-$  complementation assays indicate that the coupling defect of cardiac adenylate cyclase in the cardiomyopathic hamster is due to an abnormality in the cardiac  $\alpha G_s$  protein as the coupling defect persisted in  $cyc^-$  membranes, which contain excess amounts of normal  $\beta$ -adrenergic receptors,  $\alpha G_i$ , and  $\beta\gamma$ -subunit and normal catalytic adenylate cyclase. In addition, the inability of forskolin and guanine nucleotides to couple the cardiac  $\alpha G_s$  to catalytic adenylate cyclase in the  $cyc^-$  membranes suggests that the bioactivity defect is due to inability of the hamster  $\alpha G_s$  to interact productively with catalytic adenylate cyclase.

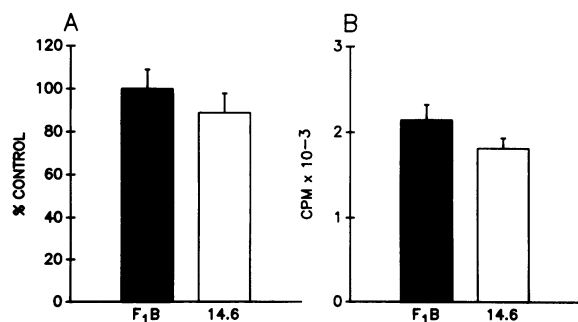
**Quantitation of  $\alpha G_s$  by immunochemical analysis.** The amount of  $\alpha G_s$  peptide on Western blots prepared from cardiac membranes of 29-d-old control and BIO 14.6 hamsters was assessed using polyclonal antisera directed against a synthetic peptide deduced from the nucleotide sequence of the carboxyl-terminal end of bovine  $\alpha G_s$  (32–34). This antisera was specific for  $\alpha G_s$  as it did not cross-react with other closely related G proteins which migrate on SDS-PAGE to 39,000–41,000 mol wt. There was a linear relation between the amount of protein applied to the immunoblot and band density on the autoradiographs when 25–100  $\mu$ g of protein was applied (Fig. 3). Alternative splicing of a single gene results in two forms of  $\alpha G_s$ : 45,000 and 52,000 mol wt (35, 36). Although the two  $\alpha G_s$  forms share the same carboxyl-terminal amino acid sequence, the ratio of their concentrations varies in different tissues. We detected only the 45,000 mol wt form on immunoblots from hamster cardiac membranes. Considering the amino acid sequence of  $\alpha G_s$  is highly conserved among mammals (12, 13, 31, 35), our finding that antisera directed against the carboxyl terminus of bovine  $\alpha G_s$  cross-reacted readily with hamster  $\alpha G_s$  is not surprising.

In contrast to our finding of different  $\alpha G_s$  bioactivities in cardiac membranes using the  $cyc^-$  complementation assay, levels of immunoreactive  $\alpha G_s$  were the same in membranes from both groups of hamsters (Fig. 4).

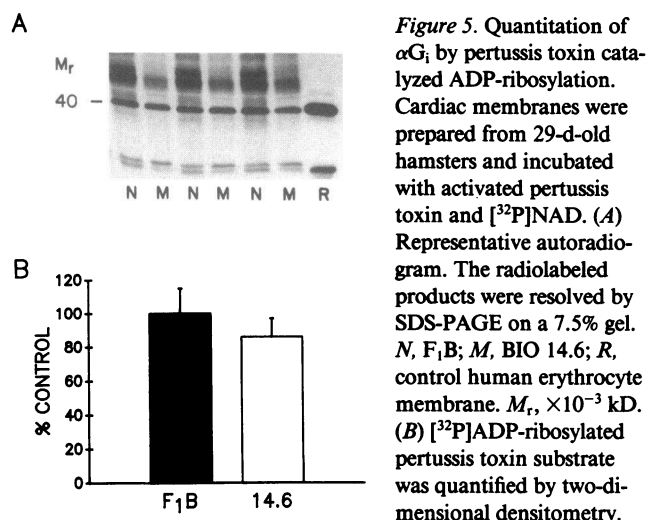


**Figure 3.** Relation between protein concentration and autoradiographic density on immunoblots. Various amounts (25–100 µg) of cardiac membranes were electrophoretically resolved by SDS-PAGE, transferred to PVDF filters, and probed sequentially with rabbit antisera specific for  $\alpha G_s$  and goat anti-rabbit  $^{125}\text{I}$ -F(ab')<sub>2</sub> (lanes 2–5). *K*, molecular weight in thousands. Lane 1 represents human erythrocyte membranes (50 µg). Autoradiographic signal intensity was measured with two-dimensional densitometry and expressed in arbitrary units.

**Quantitation of G proteins using bacterial toxin-catalyzed ADP-ribosylation.** The bacterial exotoxins from *Vibrio cholerae* and *Bordetella pertussis*, which ADP-ribosylate the  $\alpha$ -subunits of  $G_s$  and  $G_i/G_o$ , respectively, have been used previously to quantify levels of these G proteins in human hearts (5). Because increased levels of cardiac  $G_i$  can reduce the coupling efficiency of  $\beta$ -adrenergic receptors to adenylate cyclase by  $G_s$  (5), we measured  $\alpha G_i$  levels in the hearts of the normal and myopathic hamsters. Pertussis toxin catalyzed [ $^{32}\text{P}$ ]ADP-ribosylation of one major peptide band ( $\alpha G_{40}$ ). This radiolabeling was time dependent, linear over a range of membrane protein (10–125 µg), and reproducible ( $\pm 10\%$ ). Under our



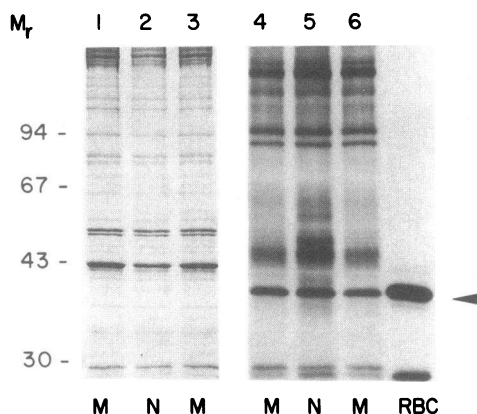
**Figure 4.** Quantitation of  $\alpha G_s$  by immunoblotting. Membrane proteins from the hearts of normal or cardiomyopathic hamster were electrophoretically resolved and transferred to support filters. After sequential incubation with rabbit antisera specific for  $\alpha G_s$  and goat anti-rabbit  $^{125}\text{I}$ -F(ab')<sub>2</sub>, binding of  $^{125}\text{I}$ -F(ab')<sub>2</sub> on the immunoblots was assessed by (A) two-dimensional densitometry of autoradiographs or (B) gamma scintigraphy. Results are (A) the mean  $\pm$  SEM ( $n = 5$ ) of three separate determinations or (B) the mean  $\pm$  SEM ( $n = 5$ ) of a representative experiment.



**Figure 5.** Quantitation of  $\alpha G_i$  by pertussis toxin catalyzed ADP-ribosylation. Cardiac membranes were prepared from 29-d-old hamsters and incubated with activated pertussis toxin and [ $^{32}\text{P}$ ]NAD. (A) Representative autoradiogram. The radiolabeled products were resolved by SDS-PAGE on a 7.5% gel. *N*, F1B; *M*, BIO 14.6; *R*, control human erythrocyte membrane. *M<sub>r</sub>*,  $\times 10^{-3}$  kD. (B) [ $^{32}\text{P}$ ]ADP-ribosylated pertussis toxin substrate was quantified by two-dimensional densitometry.

assay conditions, pertussis toxin ADP-ribosylation was complete as the further addition of [ $^{32}\text{P}$ ]NAD and activated toxin did not effect additional incorporation of  $^{32}\text{P}$  (results not shown). The amount of  $^{32}\text{P}$  incorporated into  $\alpha G_{40}$  of cardiac membranes from the BIO 14.6 hamsters was not significantly different from those of control animals (Fig. 5).

In the presence of pertussis toxin, there was ADP-ribosylation of several proteins with 47,000–58,000 mol wt in membranes prepared from normal animals (F1B) that were reduced in the membranes from myopathics (Fig. 5). However, two-dimensional densitometry (5) of multiple bands on the Coomassie Blue-stained gels (Fig. 6) did not demonstrate a difference in protein concentrations in the lanes representing normal and myopathic hearts. Furthermore, these 47,000–58,000-mol wt proteins did not bind the antisera utilized to quantitate  $\alpha G_s$ ,

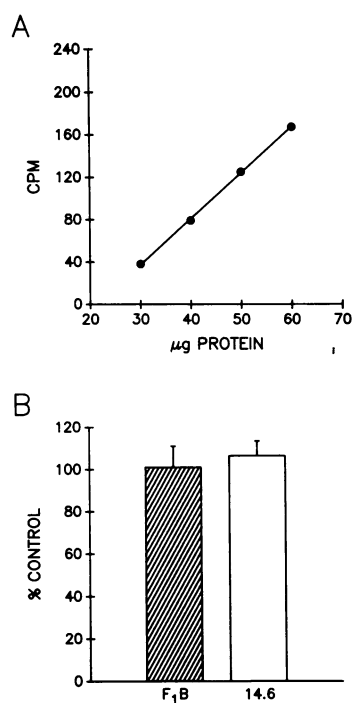


**Figure 6.** Representative autoradiograph of membranes treated with pertussis toxin. Cardiac membranes from normal (*N*) or myopathic (*M*) hamsters were incubated with activated pertussis toxin and [ $^{32}\text{P}$ ]NAD and then resolved by SDS-PAGE on a 7.5% gel. Lanes 1–3, Coomassie blue-stained gel; lanes 4–6, autoradiograph of the same gel shown in lanes 1–3 with an additional lane containing human erythrocyte membranes radiolabeled with pertussis toxin. Molecular weight markers in thousands shown at left.

(Fig. 4) or  $\alpha G_i$  (see below). Therefore, although the significance of this disparity is unclear, the levels of G proteins that are substrates for pertussis toxin were the same in cardiac membranes from the normal and myopathic animals.

Despite using reaction conditions for cholera toxin-catalyzed ADP-ribosylation which we have used successfully to ADP-ribosylate  $\alpha G_s$  in membranes from human heart (5), hamster erythrocytes and brain, rabbit heart, and dog heart (Fig. 7), we were unable to effect ADP-ribosylation of  $\alpha G_i$  by cholera toxin in cardiac membranes from either the control or the BIO 14.6 hamsters. The addition of small amounts of detergent to the reaction mixture, which has been used to allow pertussis toxin-mediated ADP-ribosylation of G proteins in sperm membranes (37), did not alter our inability to effect ADP-ribosylation. It is unlikely that this disparity is due to absence of ADP-ribosylation factor (38) in the hamster cardiac membranes, as the addition of crude ADP-ribosylation factor present in cyc<sup>-</sup> S49 cell membranes did not affect the results. The reasons for our inability to ADP-ribosylate  $\alpha G_i$  in hamster heart with cholera toxin remain unclear. This inability to adequately ADP-ribosylate  $\alpha G_i$  in the presence of cholera toxin has been found in another laboratory (Bilezikian, J. P., personal communication).

**Quantitation of  $\alpha G_i$  by immunochemical analysis.** To confirm the results of the pertussis toxin-catalyzed ADP-ribosylation, we also measured the relative amounts of  $\alpha G_i$  on Western blots prepared from cardiac membranes using an affinity-purified antibody from polyclonal antiserum raised using a synthetic peptide identical to a portion of the amino acid sequence of mouse  $\alpha G_i$  (12). In cardiac membranes, this affinity-purified antibody detected a 38,000-mol wt protein which comigrated with a protein detected by this same antibody in a preparation of partially purified G protein from bovine brain. In addition, the protein detected by the  $\alpha G_i$  antibody comigrated with a protein recognized by a polyclonal antiserum raised to a 10-amino acid synthetic peptide corresponding to a region of rat  $\alpha G_i$  common to  $\alpha G_i$ -1 and  $\alpha G_i$ -3 (Feldman, A. M., and C. Van Dop, unpublished observations). Furthermore, there was a linear relation between the amount of membrane protein applied to the immunoblot and <sup>125</sup>I bound on the Western blot (Fig. 8). The relative amount of immunodetectable  $\alpha G_i$  in cardiac membranes from BIO 14.6 animals was the same as



The results are the mean  $\pm$  SEM ( $n = 5$  animals) of three separate experiments.

that in the normal F<sub>1</sub>B animals (Fig. 8). These results with the affinity-purified antibody confirmed the data obtained with pertussis toxin-catalyzed ADP-ribosylation.

**Northern blot and dot blot analysis.** Under the high-stringency conditions used, Northern blot analysis revealed a single RNA band (1.8 kb) on blots of size-fractionated RNA prepared from hearts of both control and BIO 14.6 hamsters (Fig. 9). Using cDNA encoding the  $\alpha$ -subunit of G protein from a different mammalian species to probe RNA blots diminishes cross-hybridization of the probe to RNA encoding homologous G proteins (Levine, M. A., personal communication). Dot blots prepared using cardiac RNA from control and BIO 14.6 animals and probed with radiolabeled  $\alpha G_s$  cDNA re-

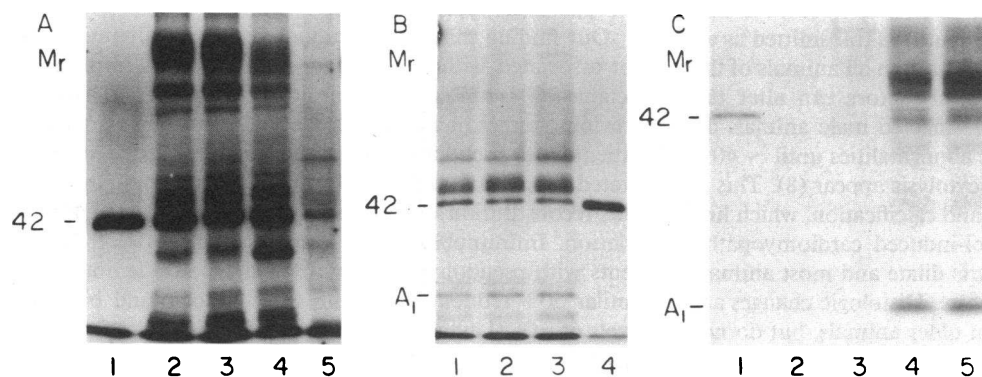
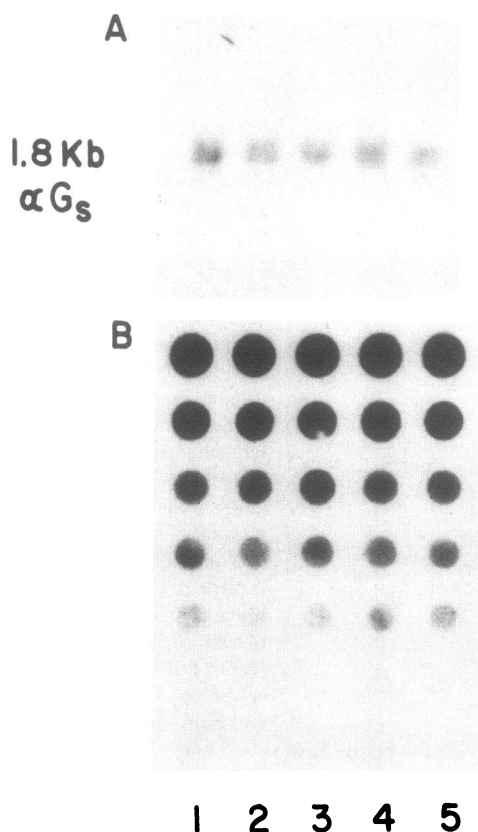


Figure 7. Autoradiograph of membranes treated with cholera toxin. Membranes prepared from tissues of 29-d-old hamsters were incubated with activated cholera toxin and [<sup>32</sup>P]NAD before fractionation using SDS-PAGE in a 7.5% gel. Molecular weight marker in thousands shown at left in each panel. Individual lanes contained 75 μg of membranes prepared from: A1, human erythrocytes; A2, human erythrocytes without cholera toxin addition; A3, cyc<sup>-</sup> S49 lymphoma membranes (which lack  $\alpha G_s$ ); A4, F<sub>1</sub>B heart without cholera toxin addition; A5, F<sub>1</sub>B heart.

erythrocytes; A5, rabbit heart; B1, F<sub>1</sub>B brain; B2, F<sub>1</sub>M brain; B3, BIO 14.6 brain; B4, human erythrocytes; C1, human erythrocytes; C2, human erythrocytes without cholera toxin addition; C3, cyc<sup>-</sup> S49 lymphoma membranes (which lack  $\alpha G_s$ ); C4, F<sub>1</sub>B heart without cholera toxin addition; C5, F<sub>1</sub>B heart. A<sub>1</sub> indicates the ADP-ribosylated cholera toxin subunit.





**Figure 9.** mRNA encoding  $\alpha G_s$  in hearts from F<sub>1</sub>B and BIO 14.6 hamsters. (A) Northern blot of size fractionated total RNA (10  $\mu$ g) from hearts of F<sub>1</sub>B hamsters (lanes 1, 3, and 5) or hearts of BIO 14.6 hamsters (lanes 2 and 4). (B) Representative dot blot probed under high-stringency conditions.

vealed no significant difference in mRNA levels encoding  $\alpha G_s$  (F<sub>1</sub>B, 103 $\pm$ 8% of control; BIO 14.6, 93 $\pm$ 8%,  $n = 11$ ).

## Discussion

The cardiomyopathic Syrian hamster (BIO 14.6) has been widely studied as an animal model for congestive heart failure in humans (39); however, the biochemical alterations responsible for development of cardiac failure remain controversial. The development of this cardiomyopathy is transmitted as an autosomal recessive trait and is expressed in all animals of the affected strains although nutritional factors can alter the course of the disease. Hearts from affected male animals do not demonstrate light microscopic abnormalities until  $\sim 40$  d of age when multiple foci of myocytolysis appear (8). This is followed by healing with sclerosis and calcification, which histologically resembles isoproterenol-induced cardiomyopathy in the rat (40). Inevitably, the hearts dilate and most animals die with frank congestive heart failure. Histologic changes are also observed in skeletal muscle of older animals, but do not lead to extreme muscle atrophy and contractures. Numerous biochemical changes have been described in hearts from this cardiomyopathic hamster. Most of these changes are detected only in the late stages of the disease (10, 41, 42). However, young animals have abnormally increased myocardial calcium

accumulation with acute isoproterenol injection (43) and elevated cardiac calcium levels (44). Of particular interest is the fact that chronic treatment of animals before the onset of myocytolysis with either isoproterenol (44) or verapamil (45, 46) delays development of the cardiac abnormalities. In summary, the cardiomyopathic state can be attenuated by treatment with either adrenergic agonists or calcium channel blockers suggesting the presence of abnormalities in the metabolic pathways that regulate both calcium metabolism and adenylate cyclase activity.

We have demonstrated in the cardiomyopathic Syrian hamster (BIO 14.6) a functional abnormality of cardiac adenylate cyclase that results from a qualitative defect in  $G_s$  protein. This abnormality of  $G_s$  is specific to cardiac and skeletal muscle, occurs only in animals homozygous for the dystrophic trait, and is present before development of characteristic light microscopic abnormalities. It is unlikely that the inability of detergent extracts from the BIO 14.6 animals to reconstitute adenylate cyclase activity in the  $cyc^-$  membranes could be due to a change in the activity of either  $\alpha G_i$  or free  $\beta\gamma$ -subunits as the  $cyc^-$  membrane contains an excess of both and the assay is therefore insensitive to changes in the levels of these peptides (5). The functional decrease in  $\alpha G_s$  was not associated with altered levels of either immunoreactive  $\alpha G_s$  or mRNA encoding  $\alpha G_s$ . Thus, it represents a qualitative rather than a quantitative defect. The functional alteration diminishes coupling of  $\beta$ -adrenergic receptors to adenylate cyclase and thereby may contribute to the development of the abnormal mechanical performance previously reported in these animals (47).

The recent demonstration (48, 49) that  $\alpha G_s$  can directly activate the slow, voltage-gated calcium channel in both cardiac and skeletal muscle independent of protein kinase-mediated phosphorylation suggests that altered bioactivity of  $\alpha G_s$  in the hearts of cardiomyopathic hamsters may contribute to the contractile abnormality. If activated  $\alpha G_s$  normally modulates calcium channels in muscle without normally increasing intracellular cAMP, disposal of cytoplasmic calcium will be abnormal. To survive, the cells would then need to sequester this increased intracellular calcium into subcellular organelles resulting ultimately in the classical pathologic abnormality seen in these animals. This hypothesis is supported by *in vivo* studies in 30-d-old myopathic hamsters which demonstrated an enhanced accumulation of  $Ca^{2+}$  after acute injection of isoproterenol (43, 50). However, further studies will be needed to support this theory.

Our finding that the functional G protein alteration was not associated with a quantitative difference in immunodetectable  $\alpha G_s$  is unique to the cardiomyopathic Syrian hamster. Previous studies in a canine model of heart failure (6) and in patients with pseudohypoparathyroidism type Ia (51) demonstrated functional alterations in  $\alpha G_s$  as measured by either  $cyc^-$  reconstitution or by cholera toxin-catalyzed ADP-ribosylation. Immunoblotting studies in erythrocytes from patients with pseudohypoparathyroidism type Ia demonstrated similar 50% reductions of both immunologic and bioactive levels of  $\alpha G_s$  (Levine, M. A., personal communication). The heritable diminution of  $\alpha G_s$  that occurs in pseudohypoparathyroidism type Ia affects all tissues and has not been reported to be associated with cardiomyopathy. Therefore, our demonstration that diminished  $\alpha G_s$  bioactivity is specific to cardiac and skeletal muscle suggests that the defect is related to the

genetic lesion in cardiomyopathic hamsters that affects only these two tissues.

It is unlikely that a generalized loss in membrane components, inflammatory or fibrotic changes, or decreased protein yield accounts for the changes demonstrated in the present study. There was no evidence of a pathologic abnormality in the 29-d-old hamsters; heart weights were equivalent in the normal and myopathic animals; and membrane markers including  $\beta$ -receptor number and levels of the 40,000-mol wt pertussis toxin G protein substrate were not significantly altered in the BIO 14.6 animals when compared with controls.

Since the promoter region of the  $\alpha G_s$  gene lacks a typical TATA box or CAAT box and has multiple transcriptional initiation sites (52), control of expression of the  $\alpha G_s$  gene is probably the same as other housekeeping genes: at the level of transcription. Therefore, the finding that steady-state levels of the message encoding  $\alpha G_s$  were not changed in the cardiomyopathic hamster supports the finding that  $\alpha G_s$  protein concentration is not altered. The alteration in functional activity of the  $\alpha G_s$  peptide likely results from either an alteration in the primary structure of the peptide or a difference in posttranslational modification. As the animals heterozygous for the cardiomyopathic trait did not demonstrate altered  $G_s$  activity, it is unlikely that a point mutation in the  $\alpha G_s$  gene accounts for our results. Alternate processing of the  $\alpha G_s$  transcript has been demonstrated (36); however, the functional significance of the resulting slight differences in amino acid sequence remains unclear (2). Cholera toxin can posttranslationally modify  $\alpha G_s$  activity by catalyzing the covalent addition of an ADP-ribose moiety to a specific arginine residue (53) on  $\alpha G_s$  (12). Additionally, cellular ADP-ribosyltransferases specific for arginine residues have been identified in eukaryotes (54), and crude cardiac membranes contain an apparently enzymatic, NAD-dependent activity that enhances adenylate cyclase and is inhibited by coincubation with arginine (55). We therefore postulate that a heritable loss of an enzyme or protein required for tissue-specific processing of transcripts or for covalent posttranslational modulation of  $\alpha G_s$  activity in muscle tissue may explain our findings in the cardiomyopathic hamster. The presence of several pertussis toxin substrates which are present in cardiac membranes of myopathic animals at considerably lower concentrations than in membranes from normal hamsters may provide clues to the identity of this protein.

In summary, we have demonstrated a functional defect in  $\alpha G_s$  of cardiac and skeletal muscle in the cardiomyopathic Syrian hamster (BIO 14.6). This defect is demonstrable before pathologic abnormalities develop in the heart and is not associated with a change in immunochemically detectable  $\alpha G_s$ .

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