Experimental Issues in Assessment of G Protein Function in Cardiac Disease

Arthur M. Feldman, MD, PhD

In 1971, Rodbell and coworkers\(^1\) found that the ability of an effector to promote synthesis of the second-messenger cyclic AMP (cAMP) by the enzyme adenyl cyclase was dependent on the presence of guanyl nucleotides. This landmark finding led to the discovery of guanine nucleotide–binding proteins, or G proteins, in the late 1970s.\(^2-4\) We now know that the G proteins are ubiquitous in eukaryotes and compose a large family of highly homologous proteins that couple nearly 100 different receptors with effector enzymes (see reviews in References 5–8). Two G proteins that have undergone extensive investigations are the stimulatory G protein (G\(_s\)) and the inhibitory G protein (G\(_i\)) that couple receptors with stimulation or inhibition of adenyl cyclase, respectively. Effector-stimulated activation of G proteins can also mediate modification of ion channel activity independent of cAMP synthesis.\(^7\)

There is direct evidence that organ-specific alterations in G protein function contribute to the pathophysiology of human disease. The florid diarrhea associated with Vibrio cholerae bacteria is the result of irreversible activation of G\(_i\); an inherited mutation in the G\(_i\) gene is responsible for at least some cases of Albright’s hereditary osteodystrophy,\(^9\) and some human endocrine tumors harbor oncogenic mutations in the \(a\)-subunits of various G proteins.\(^10\) Because the beat-to-beat control of cardiac contractility is mediated by the receptor–G protein–adenyl cyclase complex,\(^11,12\) recent interest has focused on the role of G proteins in heart disease in both humans and animal models. However, the exact role of the different G proteins in cardiac disease remains a contentious issue. Recent reviews\(^13,14\) have discussed the biochemistry and molecular biology of cardiac G proteins. This communication will clarify some of the experimental issues relating to G proteins and cardiovascular research by discussing the strengths and limitations of the technology available for evaluating G proteins, defining our present understanding of the role of G proteins in cardiac disease, and discussing the potential impact of new technology on our understanding of cardiac signal transduction.

Measurement of G Protein Function and Quantity

As seen in Table 1, numerous techniques have been used to assess both the function and quantity of G proteins in myocardial tissues. Because each of these methods has both advantages and disadvantages, it is important to understand their biochemical bases as well as their advantages and limitations before assessing the significance of laboratory investigations of G protein function.

Adenylyl Cyclase Activity

The functional activities of some G proteins (G\(_s\) and G\(_i\)) can be assessed by measuring effector-stimulated adenyl cyclase activity in particulate fractions of cardiac membranes. With the use of modifications of a technique first described by Salomon,\(^15\) the assay measures the conversion of \(\alpha\)-\(^32\)P ATP to \(\alpha\)-\(^32\)P cAMP. Radiolabeled ATP is separated from radiolabeled cAMP by Dowex-alumina chromatography, and enzyme activity is expressed as the amount of cAMP synthesized per minute per milligram of protein. The G-stimulatory pathway can be assessed by measuring adenyl cyclase activity in the presence of adrenergic agonists such as isoproterenol; effectors that activate G proteins, including fluoride and the nonhydrolyzable GTP analogues GppNHp and GTP\(_{\gamma}\); and direct enzyme agonists such as forskolin or Mn\(^{2+}\). \(\beta\)-Adrenergic agonist–stimulated adenyl cyclase activity is dependent on receptor density and affinity and therefore provides information regarding only the more distal portions of the receptor–adenyl cyclase complex when receptor K\(_D\) and \(\beta_{\text{MAX}}\) are normal. Fluoride can activate adenyl cyclase without dissociating the G more heterotrimer.\(^16\) This is supported by the fact that fluoride stimulates adenyl cyclase activity without affecting high-affinity binding of \(\beta\)-adrenergic receptor agonists\(^17\) and that fluoride-stimulated activity under appropriate conditions is not attenuated by inhibition of \(\alpha\)G\(_i\) activity with pertussis toxin.\(^18\) Therefore, activation of \(\alpha\)G\(_s\) by fluoride masks the inhibitory pathway. Forskolin acts synergistically with guanine

The opinions expressed in this article are not necessarily those of the editors or of the American Heart Association.

From the Departments of Medicine and Physiology, The Johns Hopkins University School of Medicine, Baltimore, Md.

Supported in part by National Institutes of Health grant HL-39719 and an Established Investigatorship from the American Heart Association.

Address for correspondence: Arthur M. Feldman, MD, PhD, Division of Cardiology, Hunterian 405, 725 North Wolfe Street, The Johns Hopkins University School of Medicine, Baltimore, MD 21205.
<table>
<thead>
<tr>
<th>Method</th>
<th>Use</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| Effector-stimulated adenyl cyclase | Functional measure | Not quantitative  
Preparation dependent  
Basal activity affected by tissue catecholamines, guanine nucleotides, and posttranslational modifications of αGs |
| Bacterial toxin-catalyzed ADP-ribosylation | Quantification of toxin substrates | Affected by endogenous ADP-ribosylation, β-subunits, and receptor-effector coupling  
Labor intensive and requires meticulous attention to detail  
Accurate analysis of autoradiograms requires two-dimensional densitometry  
Quantification is relative.  
αGi subspecies are difficult to resolve by sodium decyl-polyacrylamide gel electrophoresis  
Electrophoretic resolution of homologous peptides is difficult.  
Presence of undefined species with high homology precludes definitive identification.  
Antibodies may cross-react with homologous proteins  
Limitations of autoradiographic quantification as above |
| Immunoblot | Quantification of αG, β, and γ subunits | Bioassay  
Measures only functional αGς  
May be affected by βγ-subunit contamination  
Messenger RNA levels may not reflect levels or activity of gene product.  
Undefined species will not be recognized.  
Identity of product must be verified.  
Internal controls are mandatory. |
| Reconstitution | Quantification and functional activity |  |
| Quantitative polymerase chain reaction | Sensitive and specific quantification of steady-state levels of messenger RNA |  |

G, G protein; Gι, inhibitory G protein.

nucleotides to stimulate adenyl cyclase. GppNHp-stimulated adenyl cyclase activity represents a balance of the activities of αGi and αGγ and can therefore suggest an increase in αGi activity when adenyl cyclase activity is increased in the presence of GppNHp but not with fluoride. Adenyl cyclase activity can also be assessed in the presence of effectors of the inhibitory G pathway such as A1-adenosine agonists; however, these assays require alterations in the assay conditions.

Although adenyl cyclase assays are sensitive and specific, several important caveats must be kept in mind when assessing the results of adenyl cyclase measurements. First, the adenyl cyclase enzyme is extremely labile. Therefore, differences in preparative techniques can result in substantial differences in basal activities. Second, basal levels of catecholamines, guanine nucleotides, and Mg⁺ can substantially alter the degree of stimulation of adenyl cyclase activity. Finally, the addition of exogenous guanine nucleotides to a membrane preparation in which αGγ is in the associated state due to decreased receptor-αGi coupling may shift the ratio of αGγ-βγ heterotrimer to free αGγ toward dissociation. This shift would result in attenuation of differences in effector-stimulated adenyl cyclase activity that might be evident in the absence of GTP.

**Bacterial Toxin–Catalyzed ADP-Ribosylation**

The ability of specific bacterial toxins to catalyze the covalent modification of selected G proteins provided a specific and relatively inexpensive method with which to quantify G proteins. Cholera toxin catalyzes a reaction in which NAD⁺ is hydrolized to ADP-ribose and nicotinamide and the ADP-ribose moiety is covalently bound to specific amino acids on αGi, αGo, and retinal transducin (which mediates stimulation of cyclic GMP phosphodiesterase by photorhodopsin). This reaction stabilizes the GTP-bound conformation of αGi and decreases its intrinsic GTPase activity, thereby producing continuous activation. Pertussis toxin catalyzes an analogous reaction, resulting in attachment of ADP-ribose moieties to different amino acid residues on the α-chains of transducin, Gαi, and Gαs. In contrast to cholera toxin, pertussis toxin inhibits the functional activity of αGi and in vivo treatment with pertussis toxin is associated with a decline in G protein β-subunits. By using [32P]NAD as a substrate, peptides that are substrates for cholera or pertussis toxin can be visualized after gel electrophoresis under denaturing conditions and subsequent autoradiography. Although intuitively useful, quantification of αG proteins by ADP-ribosylation has limitations as bacterial...
toxins may ADP-ribosylate only αGs in the appropriate conformation to accept an ADP-ribose moiety. Furthermore, numerous factors affect the ability of a bacterial toxin to ADP-ribosylate αG peptides: The quantity of βγ-subunits can modulate ADP-ribosylation of pertussis toxin substrates;31; the presence of endogenous ADP-ribosylation factors has been shown to modify cholera toxin–catalyzed ADP-ribosylations,32 whereas specific inhibitors can alter pertussis toxin–catalyzed ribosylations33; the degree of receptor–effector interaction can modulate pertussis toxin–catalyzed reactions34; and phosphorylation of G protein modulates subsequent ADP-ribosylation by pertussis toxin.35 Furthermore, as pertussis toxin ADP-ribosylates αG only in its heterotrimeric (GDP-bound) form,34 an increase in pertussis toxin labeling might reflect a relative increase in total βγ-subunits rather than an increase in αG, whereas a decrease in αG may represent a decrease in βγ-subunits. To exclude these possibilities, investigators have assessed the effect of added βγ-subunits on pertussis toxin–catalyzed ADP-ribosylation.

Resolution of the different pertussis toxin–sensitive G proteins in one-dimensional sodium decyl–polyacrylamide gels is often poor, but resolution can be improved by prior alkylation of the samples with N-ethylmaleimide36 or by using low bisacylamide concentrations.37 However, only two-dimensional electrophoresis provides optimal separation of the αG1 peptides. Pertussis toxin–catalyzed ADP-ribosylation is a relatively sensitive technique; a 30% difference in the level of pertussis toxin substrates can be detected when the sample size is adequate. Measurements in smaller sample sizes are subject to experimental error. Preliminary experiments must demonstrate that radiolabeling of pertussis toxin substrates is complete, and the response to increasing amounts of membrane protein must be linear within the range of protein concentrations used in each experiment. Furthermore, accurate assessment of the amount of radiolabel incorporated into each peptide of interest is critical. In our experience, two-dimensional computer-assisted densitometry provides the most accurate measure of [32P]NAD incorporation. Alternatively, we have also produced a template from the autoradiogram and used it to cut out the bands of interest from the dried gel. These bands can then be counted by Cerenkov counting.

Immunochromical Detection of G Peptides

Initial attempts to produce polyclonal antisera selective for individual cholera and pertussis toxin–sensitive G proteins by immunizing rabbits with purified αG peptides proved difficult (see review in Reference 38). This was not surprising in view of the high degree of homology between these αG peptides. In 1986, Mumby et al 39 first generated a series of antipeptide antisera that were raised against synthetic peptides corresponding to the deduced amino acid sequences of various αG peptides. Similarly, antibodies raised against purified βγ-subunits have been used to quantify peptide levels with an immunosassay,40 and synthetic peptides corresponding to the deduced amino acid sequences common to the β35- and β36-peptides have been used to quantify immunodetectable peptides.41 However, the ability to produce polyclonal antisera that unequivocally distinguish the pertussis toxin–sensitive G proteins αG1-1 and αG1-3 is still limited because of the high degree of conservation among the αG peptides and the limited number of highly charged and potentially antigenic regions. Furthermore, definitive identification of immunodetectable peptides requires that their affinity for and coaggregation with purified protein be demonstrable. Unfortunately, purified αG peptides are not readily available. In addition, the structural similarities of these proteins impedes their electrophoretic resolution, and the possible existence of multiple undefined species42 may cast doubt over the homogeneity of a given G protein preparation.

Numerous investigators have used recombinant αG peptides to assess the specificity of selected antisera as well as the affinity of those antisera for particular peptides.41,43 However, this approach is limited as the lack of posttranslational modifications in the recombinant proteins may alter both their electrophoretic mobility as well as their affinity for antibody. Variations in electrophoretic systems and gel concentrations will result in small differences in the relative molecular weights of specific αG peptides. For example, immunodetectable αG1 has been reported to have a molecular weight between 40 and 43 kd.45 Therefore, although immunochromical detection can be used to quantify proteins with a specific relative molecular weight, it is difficult to definitively identify the immunodetected peptide found in crude membrane fractions. In addition, the limitations of analysis of autoradiographs that were discussed for ADP-ribosylation experiments also apply to immunoblot analyses.

Reconstitution Studies

The isolation by Bourne and his colleagues46 of a mutant form of the S49 lymphoma cell line that did not synthesize cAMP in response to β-adrenergic agonists and the subsequent demonstration that this mutant lacked a cholera toxin substrate47 provided a tool for quantifying G protein activity. This cya cell line lacks immunodetectable levels of both αG and the messenger RNA (mRNA) encoding αG.48 Therefore, the ability of an adrenergic agonist to stimulate cAMP synthesis is directly related to the amount of exogenous αG reconstituted with the cya membranes. This technique provides a useful measure of αG function. Unfortunately, similar mutants of pertussis toxin–sensitive G proteins are not available. However, several laboratories have attempted to create cells functionally deficient in αG by pretreating cells with pertussis toxin.49 These cells can be used to assess the ability of receptors to interact with the exogenous G proteins, and successful reconstitu-
tion of G protein activity should restore high-affinity agonist binding to the receptor, produce agonist-stimulated high-affinity GTPase activity, and restore receptor-mediated alterations in cAMP synthesis. Unfortunately, the inhibitory receptors appear to be promiscuous, showing little selectivity for the pertussis toxin–sensitive G proteins under reconstitution conditions.18 In addition, these studies are limited by the lack of homogenous preparations of αG,-subtypes and the contamination of these preparations by variable amounts of By; therefore, they provide qualitative rather than quantitative information.

Measurement of Steady-State Levels of αG mRNAs

The cloning and sequencing of the complementary DNAs (cDNAs) encoding αG, and the αG,-subtypes provided the ability to measure steady-state levels of αG mRNAs in normal and diseased heart. Because regulation of gene expression is often at the level of transcription, it was hypothesized that mRNA levels would reflect the quantity of the gene product. The high degree of interspecies conservation for nucleotide sequences encoding αG, subspecies provided an impediment to measurements of mRNA levels using standard hybridization techniques because considerable cross-hybridization can occur even under highly stringent conditions. For example, nucleotide sequences of human αG,-2,50 αG,-3,50 and αG,51 share approximately 92% sequence identity with the homologous αG sequences of the rat,52 and comparisons of coding nucleotide sequences of the different αG sequences in the rat indicate 85–90% sequence identity.51 By contrast, comparison of the coding nucleotide sequences for a human αG with a non-complementary rat αG protein reveals far less homology (less than 75% sequence identity). Therefore, the rat cDNA probes could be used to quantify human mRNA for the αG, peptides without cross-hybridization to homologous mRNAs when experiments are performed under appropriate (high stringency) conditions. However, these studies are time consuming, necessitate the acquisition of relatively large (gram) quantities of tissue, and require the availability of full-length cDNAs complementary to the mRNA of interest.

The polymerase chain reaction (PCR) has recently been used to assess mRNA levels in small samples of myocardium obtained from patients at the time of endomyocardial biopsy.53 This technique not only obviates the technical problems associated with Northern blot hybridizations but also allows measurements to be made in small samples rather than in the large (gram quantities) amounts of myocardium required by older techniques.54,55 Total RNA is extracted from 3–5-mg samples of human heart and reverse-transcribed to cDNA. In the presence of selected oligonucleotide primers, PCR can be used to amplify the cDNAs encoding multiple proteins of interest, including the αG proteins. By including a known amount of a control RNA in the reverse transcription and PCR reactions, the amount of mRNA in a sample can be assessed in relation to the amount of control product.56 Using quantitative PCR, we demonstrated selective alterations in gene expression in failing human heart that can be reversible (PW Ladenson, AM Feldman; unpublished observations). Therefore, PCR may provide a useful tool for measuring the effects of pharmacological therapy on the expression of the proteins of the receptor–G protein–adenylyl cyclase complex.

Although measurements of steady-state levels of mRNAs encoding the G proteins provide important insight into the basis for functional protein modifications, it is critical to note that both the function and amount of the αG proteins can be altered without a change in the levels of their respective transcripts.57 Conversely, increased levels of mRNA may not be reflected in increased levels or functional activity of the gene product. Regulation of the functional activity of a protein can occur at the level of translation as well as after translation, and therefore steady-state levels of mRNA may not reflect the levels of gene product. Thus, it is important that mRNA measurements be correlated with functional measures as well as quantitative evaluation of the gene products.

G Protein Function in Cardiovascular Disease

Animal Models of Heart Disease

Table 2 summarizes investigations that have demonstrated an association between cardiovascular disease and altered G protein function in experimental animals. Several points merit attention. First, few studies have measured both quantitative and functional parameters. Therefore, it is difficult to determine whether an increase in quantity correlates with an increase in function or, alternatively, whether an increase in function results from an increase in the quantity of a specific G protein. This is an important consideration as function and quantity may be unrelated in a given biological situation. A second caveat is that alterations in either G protein function or quantity are both intervention and species dependent. Thus, one cannot view alterations in a specific model as being generic.

Numerous studies have demonstrated that G protein changes are intervention dependent. Congestive failure after aortic banding in the dog,58,59 volume overload hypertrophy in the pig,60 hereditary myopathy in the Syrian hamster,61 and chronic ischemia62 appear to be associated with decreased functional activity (although not necessarily quantity) of αG,. In contrast, cardiac denervation,63,64 myocardial changes associated with hypothyroidism,41 and chronic adrenergic stimulation65,66 are all associated with increased levels of αG,. Furthermore, chronic dynamic exercise in pigs resulted in an increase in the amount of αG, a change that was discordant with β-receptor number.67 Infection with Trypanosoma cruzi resulted in a decrease in both pertussis and cholera toxin substrates in mice,68 whereas chagasic cardiomyopathy in dogs appears to be associated only with a change in αG,69
Receptor effector coupling is also altered in right ventricle from dogs with pulmonary artery banding, left ventricular myocardium from turkeys with hereditary cardiomyopathy, and ventricular myocardium from spontaneously hypertensive rats. However, the relation of these changes to levels and functional activity of the G proteins remains unclear because only one experimental technique was used to assess G protein function.

The interventional model that has been studied most extensively is the canine model with aortic banding. Initial hypertrophy in these animals is associated with a 30% decrease in functional G, and a similar decrease in the levels of the mRNA encoding αG. However, with the onset of left ventricular failure, there is a further decrement in adenylyl cyclase activity independent of additional changes in G, function or αG mRNA levels. Therefore, alterations in receptor-effector coupling are time dependent with a given intervention, and the modulating systems are complex. The biological significance of the 30% change in mRNA levels in the hypertrophied myocardium is unclear.

That species diversity plays a critical role in defining the G protein response to cardiovascular disease is demonstrated by studies of G protein function in different strains of myopathic Syrian hamsters. The BIO 14.6 or “hypertrophic” myopathic hamster has a well-described life cycle with myocytolysis at approximately 40 days of age, cellular hypertrophy by 100 days of age, and frank dilatation at approximately 250 days of age. In contrast, the “dilated” myopathic hamster (BIO 53.58, now referred to as TO), which is a spontaneous mutation of the BIO 14.6 strain, demonstrates ventricular dilatation at 100 days of age without evidence of preexisting hypertrophy. The BIO 14.6 animals demonstrate a substantial decrease in the functional activity of αG at 28 days of age.

### Table 2. Alterations in G Protein Function and Quantity in Experimental Models of Cardiovascular Disease

<table>
<thead>
<tr>
<th>Species</th>
<th>Intervention</th>
<th>Effector-stimulated AC</th>
<th>β-AR</th>
<th>αG</th>
<th>αGi</th>
<th>Reference</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog</td>
<td>Aortic banding</td>
<td>↓ ↓ ↓</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>Vatner et al&lt;sup&gt;59&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Longabaugh et al&lt;sup&gt;58&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P.A. banding</td>
<td>↓ ↓ ↓</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>Fan et al&lt;sup&gt;57&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Parasympathectomy</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>Hodges et al&lt;sup&gt;63&lt;/sup&gt;</td>
<td>↑ Muscarinic receptor</td>
</tr>
<tr>
<td></td>
<td>Denervation</td>
<td>↔ ↔ ↓</td>
<td>↔</td>
<td>↑</td>
<td>↓</td>
<td>Hershberger et al&lt;sup&gt;64&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ischemia</td>
<td>↓ ↓ ↓</td>
<td>↓</td>
<td>↓</td>
<td>↔</td>
<td>Karliner et al&lt;sup&gt;62&lt;/sup&gt;</td>
<td>Reversal with β-blockade</td>
</tr>
<tr>
<td>Pig</td>
<td>Chronic exercise</td>
<td></td>
<td>↑</td>
<td></td>
<td></td>
<td>Hammond et al&lt;sup&gt;60&lt;/sup&gt;</td>
<td>Iso ED&lt;sub&gt;50&lt;/sub&gt; ↓</td>
</tr>
<tr>
<td>Turkey</td>
<td>Hereditary myopathy</td>
<td>↓ ↓</td>
<td></td>
<td></td>
<td></td>
<td>Staley et al&lt;sup&gt;73&lt;/sup&gt;</td>
<td>↓ Iso responsiveness</td>
</tr>
<tr>
<td>Syrian hamster</td>
<td>Hypertrophic 28 days</td>
<td>↓ ↓</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>Kessler et al&lt;sup&gt;61&lt;/sup&gt;</td>
<td>Also seen in skeletal muscle</td>
</tr>
<tr>
<td></td>
<td>154 days</td>
<td>↓ ↓</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>Katoh et al&lt;sup&gt;74&lt;/sup&gt;</td>
<td>Only αG&lt;sub&gt;2&lt;/sub&gt;-2 ↓, not αG&lt;sub&gt;3&lt;/sub&gt;-3 messenger RNA</td>
</tr>
<tr>
<td></td>
<td>224 days</td>
<td>↑</td>
<td></td>
<td></td>
<td></td>
<td>Sen et al&lt;sup&gt;76&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dilated 30 days</td>
<td>↔ ↓ ↓</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>Feldman et al&lt;sup&gt;75&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 days</td>
<td>↓ ↓ ↓</td>
<td>↔</td>
<td>↔</td>
<td>↓</td>
<td>Feldman et al&lt;sup&gt;73&lt;/sup&gt;</td>
<td>↓ Iso contractility</td>
</tr>
<tr>
<td>Mouse</td>
<td>Trypanosoma cruzi</td>
<td>↓ ↓</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>Morris et al&lt;sup&gt;68&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>Spontaneous hypertension</td>
<td>↓ ↓</td>
<td></td>
<td></td>
<td></td>
<td>Anand-Srivastava&lt;sup&gt;72&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

AC, adenylyl cyclase; Iso, isoproterenol; F, fluoride; GppNHz, 5-guanylyl inodophosphate; β-AR, β-adrenergic receptor; CTS, cholera toxin substrate; Pts, pertussis toxin substrate; IαG, immunodetectable αG; IαGi, immunodetectable αGi; Iβ, immunodetectable β-subunit of G protein; Cyc<sup>-</sup>-, reconstitution studies with membranes genetically deficient in αG<sub>i</sub>; P.A., pulmonary artery.

Arrows indicate statistically significant changes. Changes of more than twofold are indicated by double arrows.
before the onset of phenotypic changes. This decrease is not associated with changes in levels of immunodetectable $\alpha G_i$ or in steady-state levels of the mRNA encoding $\alpha G_i$. However, in older animals (154 days old), steady-state levels of the mRNA encoding $\alpha G_i$ are decreased.74 In contrast, a spontaneous mutation of the BIO 14.6 strain that does not develop hypertrophy and demonstrates dilatation at 100 days of age (BIO 53.58, or TO) was found to have a functional defect in $\alpha G_i$ only at the time of dilatation.75 The levels of a pertussis toxin-sensitive substrate are increased in the older BIO 14.6 animals,76 whereas immunodetectable levels of $\alpha G_i$ are unchanged in both young and old BIO 53.58 hamsters.75 Therefore, although the structures of G proteins are highly conserved among eukaryotes, regulatory mechanisms may differ substantially among closely related species. It is notable that the decrease in $\alpha G_i$ in the 100-day-old BIO 53.58 hamsters is associated with a diminished contractile response to catecholamines in the absence of changes in adrenergic receptor number. However, because this hereditary cardiomyopathy is associated with other cellular changes (i.e., decreased Ca$^{2+}$ channels), a cause-and-effect relation cannot be established.

Several pharmacological perturbations also alter $G$ protein function or quantity in heart. Halothane increases cyclic GMP synthesis via a pertussis toxin–sensitive $G$ protein,77 epidermal growth factor stimulates adenyl cyclase via a $G$-stimulatory protein that is cholera toxin sensitive,78 and interleukin-1 inhibits $\beta$-adrenergic responsiveness,79 presumably via an activation of a pertussis toxin–sensitive signal transduction protein.80 Only in the latter experiments has an alteration in cAMP synthesis been correlated with a change in cardiac morphology or mechanics.

### Human Heart Disease

Considerable attention has been given to the association between human heart muscle disease and $G$ protein function. In 1988, we demonstrated that human hearts with idiopathic dilated cardiomyopathy have increases in the levels of a pertussis toxin substrate with a relative molecular weight of 40,000.81 This increase in pertussis toxin substrate was associated with diminished adenylyl cyclase activity. Furthermore, the increase in pertussis toxin substrate and the decrease in effector-stimulated adenylyl cyclase activity appear related as inhibition of $\alpha G_i$ by pertussis toxin eliminated the differences in effector-stimulated cyclase activity in membranes from failing and nonfailing human heart. In contrast to the change in $G_i$, neither functional activity of $\alpha G_i$ as measured by cGMP recombination studies nor levels of $G_i$ as measured by cholera toxin–catalyzed ADP-ribosylation are altered during idiopathic heart failure. As seen in Table 3, other investigators have confirmed an increase in pertussis toxin substrate,82–84 a lack of change in $\alpha G_i$,85 and a decrease in guanine nucleotide–stimulated adenyl cyclase activity84,86,87 in failing human heart. Using $G_i$–specific antisera and an enzyme immunosorbent assay, Ransnas et al88 demonstrated a substantial decrease in dissociated (activated) $\alpha G_i$ in membranes prepared from patients with idiopathic dilated cardiomyopathy. However, these changes could be ac-

<table>
<thead>
<tr>
<th>Disease</th>
<th>Reference</th>
<th>Basal</th>
<th>Effector-stimulated AC</th>
<th>CTS</th>
<th>Pts</th>
<th>Ia$G_i$</th>
<th>Ia$G_i$</th>
<th>$\beta$ Cyc$^{-}$</th>
<th>$\alpha G_i$</th>
<th>$\alpha G_i$</th>
<th>$B_{MAX}$</th>
<th>$K_D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Idiopathic dilated cardiomyopathy</td>
<td>Feldman et al83,54,81,91</td>
<td></td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\uparrow$</td>
<td>$\downarrow$</td>
<td>$\uparrow$</td>
<td>$\uparrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
</tr>
<tr>
<td></td>
<td>Karliner and Scheinman86</td>
<td></td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td></td>
<td></td>
<td>$\uparrow$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Neumann et al82</td>
<td></td>
<td>$\uparrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Denniss et al87</td>
<td></td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td></td>
<td></td>
<td>$\downarrow$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Schnabel et al85</td>
<td></td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bahr et al85,84</td>
<td></td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denervated myocardium</td>
<td>Denniss et al87</td>
<td></td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischemic cardiomyopathy</td>
<td>Bristow et al82</td>
<td></td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td>$\downarrow$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AC, adenylyl cyclase; Iso, isoproterenol; F, fluoride; GppNHp, 5-guanosyl inidodiphosphate; Forsk, forskolin; $\beta$-AR, $\beta$-adrenergic receptor; CTS, cholera toxin substrate; Pts, pertussis toxin substrate; Ia$G_i$, immunodetectable $\alpha G_i$; Ia$G_i$, immunodetectable $\alpha G_i$; I$\beta$, immunodetectable $\beta$-subunit of G protein; Cyc$^{-}$, reconstitution studies with membranes genetically deficient in $\alpha G_i$; LV, left ventricle.

Arrows indicate statistically significant changes. Changes of more than twofold are indicated by double arrows.

**TABLE 3. Quantitative and Qualitative Assessment of G Protein Activity in Human Heart Disease**
counted for by the differences in the levels of catecholamines contaminating crude membranes prepared from failing and nonfailing human hearts as the studies were not performed in the presence of β-blockade.

By contrast, controversy exists regarding whether similar changes occur in circulating lymphocytes from patients with congestive heart failure. Horn et al demonstrated a substantial decrease (80%) in levels of Gi (cholera toxin substrate) in lymphocytes of patients with congestive heart failure. Maisel and colleagues were unable to find a decrease in cholera toxin substrates in lymphocytes harvested from patients with congestive heart failure compared with controls. However, these investigators did demonstrate an enhanced functional activity of pertussis toxin substrates in the lymphocytes of the heart failure patients. The reason for the discrepancy between these two studies is unclear but may be related to methodologic differences in the ADP-ribosylation techniques.

Similar controversy exists regarding immunochromatographic detection of αGi proteins in failing human heart. Bohm and colleagues reported an increase in immunodetectable αGi in myocardium obtained from a small group of patients (n=5) with idiopathic dilated cardiomyopathy compared with nonfailing controls. Although basal and GppNHp-stimulated adenyl cyclase activities were diminished in the membrane preparations from failing human heart, there was no alteration in the levels of immunodetectable β-subunits. An increase in αGi in the absence of a concomitant increase in βγ would be expected to shift the equilibrium of α-βγ dissociation toward reassociation of the heterotrimer, resulting in a decrease in the inhibitory pathway and an increase in adenyl cyclase activity. Therefore, these data are not internally consistent based on a current paradigm of how G proteins inhibit adenyl cyclase. Using two different αGi antibodies, we were unable to detect differences in immunodetectable levels of αGi5 in hearts from patients with idiopathic dilated cardiomyopathy despite finding increased levels of pertussis toxin substrate. The disparity between these two studies may be due to methodologic differences or, more likely, a difference in sample size.

Studies using Northern blot analysis demonstrated an increase in the steady-state levels of mRNA encoding both αGi and αGi in failing human heart and led us to hypothesize that enhanced transcriptional activity of the αGi proteins contributed to the increase in the functional activity of Gi. However, we have recently repeated these measurements using quantitative PCR, a more sensitive and specific technique. These studies demonstrate that αGi and αGi-3 gene expressions are unaltered in the failing heart with idiopathic dilated cardiomyopathy (unpublished observations) and suggest that earlier results were biased by normalization of the Northern blot signals to the levels of β-cytoskeletal actin in each sample. These results from quantitative PCR analysis are more consistent with the determinations of αGi protein levels in failing human heart and imply that the increase in the functional activity of Gi is the result of posttranslational modifications rather than of altered transcriptional regulation.

Additional controversy exists regarding alterations in receptor-effector coupling via Gi proteins in myocardium from patients with dilated cardiomyopathies secondary to ischemic heart disease. Bohm and co-workers reported that in contrast to hearts from patients with idiopathic dilated cardiomyopathy, membranes prepared from hearts with ischemic cardiomyopathy did not show decreases in basal or GppNHp-stimulated adenyl cyclase activity compared with measurements performed in membranes prepared from five nonfailing hearts. Similarly, they were unable to detect an increase in either pertussis toxin substrate (n=3 for control) or immunodetectable αGi in hearts with ischemic cardiomyopathy, whereas hearts with idiopathic or ischemic dilated cardiomyopathy demonstrated comparable decreases in total β-adrenergic receptor density. These studies were in marked contrast with a recent study by Bristow et al demonstrating changes in GppNHp-stimulated adenyl cyclase activity in membranes prepared from a substantial number of hearts with idiopathic (n=31) or ischemic (n=23) cardiomyopathy compared with nonfailing controls (n=20). In addition, no differences were noted between levels of pertussis toxin substrate in the two groups; however, the levels of β-adrenergic receptors were lower in the patients with idiopathic heart disease than in those with ischemic myopathy. Although receptor levels were lower in the myocardium from patients with idiopathic dilated cardiomyopathy, myocardium from ischemic hearts exhibited greater uncoupling of β-adrenergic receptors from mechanical response. In addition, left ventricular myocardium from hearts with ischemic myopathy exhibited greater subsensitivity to β-agonist-mediated adenyl cyclase stimulation than did myocardium from hearts with idiopathic myopathy, suggesting uncoupling of β-receptors from cAMP generation. Furthermore, myocardium from hearts with ischemic myocardium demonstrated less stimulation of adenyl cyclase by fluoride and forskolin but no change in manganese stimulation. These results suggest distinct pathophysiological differences between these two types of heart muscle disease that cannot be solely accounted for by differences in G protein function. The etiology of the discrepancy between the two studies is unclear but may be due to different experimental techniques used in quantification of ADP-ribosylation and immunoblot autoradiograms, marked differences in sample size, and a substantial difference in ejection fractions between the two study populations (16.5% versus 29.6%).

Conclusions

There is general agreement that many cardiac diseases can be associated with alterations in both the function and quantity of G proteins. However,
these changes are both intervention and species specific. This is clearly illustrated in the numerous studies of G protein function in congestive heart failure. The failing heart has been reported to have increased functional activity of $\alpha G$, as well as decreased functional activity of $\alpha G$. However, in all tissues studied to date, the common pathophysiological phenomenon is a decrease in the ability of the failing heart to produce cAMP in response to adrenergic signaling. It is also important to note that the functional activity of the G proteins may not correlate with their quantitative levels as posttranslational modifications can substantially alter G protein activity and stability. In addition, techniques used for both quantitative and qualitative evaluations have limitations that affect data interpretation. Therefore, it is imperative that hypotheses be based on data that reflect both the functional and the qualitative aspects of the G proteins and that at least several of the complementary but mutually exclusive techniques outlined in Table 1 be used in experiments designed to assess cardiovascular G protein function. Finally, as new G proteins are being recognized with increasing frequency, investigators must refrain from definitively identifying either immunochromically detectable proteins or ADP-ribosylation substrates without irrefutable evidence to support the identity of the peptide.

Despite the exponential increase in our understanding of the regulation of signal transduction proteins in heart disease, we do not yet know whether the alterations in G protein function in cardiac disease states represent physiologically significant changes or merely epiphenomena. In the case of the hypertrophic Syrian hamster, alterations in the function of $\alpha G$ occur before the onset of typical phenotypic changes; however, the relation between cause and effect remains unresolved. Although quantitative information will continue to be important in discerning G protein alterations in various cardiovascular diseases, it is the relevance of these changes to the pathophysiology of cardiovascular disease that poses an important experimental question for the future. The identification of regulatory elements in the promoter regions of the $\alpha G$ genes may lead to our ability to selectively turn on or turn off G protein gene expression in vivo and thereby produce models with increased G protein activity. Alternatively, the use of transgenic animals or gene transfer may provide the tools with which to selectively alter G protein gene expression in animal models. If the modest alterations in G protein activity that have been noted in both human heart disease and experimental models of heart disease are of physiological relevance, we would expect these animals with altered G protein expression to have identifiable abnormalities in cardiac pathology, contractility, and a change in adrenergic responsiveness. Therefore, although it is clear that G protein function may be altered in heart muscle disease, important information regarding the role of G proteins in cardiovascular disease remains to be discovered.

Acknowledgments

The author thanks Dr. Gerda E. Breitwieser and Dr. Paul D. Kessler for critically reviewing the manuscript.

References


Downloaded from http://circ.ahajournals.org/ by guest on September 1, 2017
Asano 38.
Jacquemin 31.
29.
26. Katada

26.

31.

30.

29.

33.

32.

31.

39.
Hara-Yokoyama M, Furuyama S: The endogenous inhibitor of the ADP-ribosylation of GTP binding proteins by pertussis toxin is present in bovine brain. Biochim Biophys Acta 1989;1038:67–71

34.

35.

36.

37.

38.

39.

40.

41.

42.

43.

44.

45.
Scherer NM, Toror M-J, Entman ML, Birnbaumer L: G-protein distribution in canine cardiac sarcoplasmic reticulum and sarcolemna: Comparison to rabbit skeletal muscle membranes and to brain and erythrocyte G-proteins. Arch Biochem Biophys 1987;259:431–440

46.

47.
Johnson GL, Bourne HR: Influence of cholera toxin on the regulation of adenylate cyclase by GTP. Biochem Biophys Res Commun 1977;78:792–796

48.

49.

50.

51.

52.

53.

54.

55.

56.

57.

58.

59.

60. Hammond HK, Ransnas LA, White FC, Bloor CM, Insel PA: Myocardial β-receptor number, pharmacological responsiveness, and Gs in volume overload hypertrophy in pigs (abstract). Circulation 1988;78(suppl II):H-163


73. Chen L, Vatner DE, Vatner SF, Hittinger L, Homey CJ: Decreased Gα mRNA levels accompany the fall in Gα and adenylyl cyclase in compensated left ventricular hypertrophy. J Clin Invest 1991;87:293–298


KEY WORDS • Point of View • G proteins

Downloaded from http://circ.ahajournals.org by guest on September 12, 2017
Experimental issues in assessment of G protein function in cardiac disease.

A M Feldman

_Circulation_. 1991;84:1852-1861
doi: 10.1161/01.CIR.84.4.1852

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1991 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/84/4/1852.citation