

Mechanisms of Impaired β -Adrenergic Receptor Signaling in $G_{\alpha q}$ -Mediated Cardiac Hypertrophy and Ventricular Dysfunction

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ABSTRACT

Targeted cardiac overexpression of the α -subunit of the heterotrimeric G protein G_q in transgenic mice evokes hypertrophy and depressed stimulation of cardiac inotropy and chronotropy by β -adrenergic receptor (β AR) agonists in vivo, which is a hallmark of many forms of experimental and human heart failure. The molecular basis of this β AR dysfunction was explored in transgenic mice overexpressing $G_{\alpha q}$ \sim 5-fold over background. Isoproterenol-stimulated adenylyl cyclase activities in myocardial membranes were significantly depressed in $G_{\alpha q}$ mice compared with nontransgenic controls (19.7 ± 2.6 versus 43.7 ± 5.6 pmol/min/mg) without a decrease in β AR expression levels. Functional coupling of both β AR subtypes was impaired. Similarly, in whole-cell patch-clamp studies, β AR stimulation of L-type Ca^{2+} channel currents was depressed \sim 75% in the $G_{\alpha q}$ mice. Cardiac β AR from these mice showed decreased formation of the active high-affinity conformation ($R_H = 29\%$ versus 62% for nontransgenic littermates), confirming a receptor- G_s -

coupling defect. Of the three candidate kinases that might impose this uncoupling by receptor phosphorylation (protein kinase A, β AR kinase, protein kinase C), only protein kinase C activity was elevated in $G_{\alpha q}$ mouse hearts. Type V adenylyl cyclase was decreased \sim 45% in these mice, consistent with decreased basal, NaF, and forskolin-stimulated enzyme activities. Although cellular G_s levels were unaltered, G_{i2} and G_{i3} were increased in $G_{\alpha q}$ mice. Pertussis toxin treatment of isolated $G_{\alpha q}$ myocytes resulted in an improvement in β AR, but not that of forskolin or NaF, stimulation of adenylyl cyclase. Thus three distinct mechanisms contribute to impaired β AR function by in vivo G_q signaling cross-talk in myocytes. Because many elements of hypertrophy and/or failure in cellular and animal models can be initiated by increased $G_{\alpha q}$ signaling, the current work may be broadly applicable to interfaces whereby modification of heart failure might be considered.

β -adrenergic receptors (β AR) are cell-surface G-protein-coupled receptors (GRK) that are expressed in the heart, mediating the positive inotropic and chronotropic effects of catecholamines. Like other adrenergic receptors, β AR function is dynamically regulated, a component of normal physiologic adaptation to maintain homeostasis. During pathologic processes, such regulation can be compensatory, or it can contribute to the pathophysiology of the condition. In either case (compensation or maladaptation), an understanding of the basis of receptor regulation in complex systems such as the failing heart may provide new insights into the events that ultimately affect phenotype, as well as potential therapeutic strategies.

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We recently reported the cardiac manifestations of 4- and 5-fold overexpression of the α -subunit of G_q in transgenic mice (D'Angelo et al., 1997). The phenotype includes myocyte hypertrophy, expression of fetal genes, and depressed ventricular function. Expression of the transgene to a greater extent, or pregnancy, results in frank cardiac failure and death (Adams et al., 1998). The development of this animal model was based on the experimental evidence that many potential mediators of hypertrophy and failure, as determined in cell-based systems and other animal models, can be evoked by enhanced G_q signaling (see *Discussion*). The $G_{\alpha q}$ model is particularly relevant from a physiologic standpoint in that the phenotype occurs in the absence of external mechanical or hemodynamic stress. Profound cardiac β AR hyporesponsiveness in vivo was also observed in the $G_{\alpha q}$ mice. Thus the current studies were undertaken to delineate the molecular basis of this β AR dysfunction as induced by en-

ABBREVIATIONS: β AR, β -adrenergic receptor; β_1 AR, β_1 -adrenergic receptor subtype; β_2 AR, β_2 -adrenergic receptor subtype; β ARK, β AR kinase; GRK, G-protein-coupled receptor kinase; PKC, protein kinase C; PKA, protein kinase A; I_{Ca} , Ca^{2+} channel current; ^{125}I -CYP, ^{125}I -cyanopindolol; NTG, nontransgenic; PAGE, polyacrylamide gel electrophoresis; HEK, human embryonic kidney.

hanced G_q signaling in the heart. The results indicate that the mechanism of impaired βAR signaling in this context is complex and involves multiple alterations in elements of the signal transduction cascade and point toward three key interfaces where interventions may be targeted.

Materials and Methods

Transgenic Mice. The creation of FVB/N transgenic mice expressing the murine α-subunit of G_q in the heart via the α-myosin heavy chain promoter has been previously described (D'Angelo et al., 1997). For the current studies, the line previously termed G_{αq}40, which expresses G_{αq} 5-fold over endogenous levels, was used for all studies along with age-matched nontransgenic mice. To confirm the critical features of the functional phenotype, additional selected studies were carried out with another transgenic line, G_{αq}25, which expresses G_{αq} 4-fold over endogenous levels (D'Angelo et al., 1997). All animals were studied at 8 to 12 weeks of age.

Adenylyl Cyclase Activities. Ventricles were homogenized with a Polytron for 10 s in cold 5 mM Tris, 2 mM EGTA buffer, pH 7.40 containing the protease inhibitors (5 μg/ml) leupeptin, phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, benzamidine, and aprotinin. Homogenates were centrifuged at 500g for 10 min at 4°C, and the pellet was discarded. The supernatant was centrifuged at 40,000g for 10 min, and the pellet was resuspended in a buffer that provided for a final concentration in the reaction of 2 mM Tris, 4.8 mM MgCl₂, 0.8 mM EGTA, pH 7.40, with the aforementioned protease inhibitors. Adenylyl cyclase activities were measured essentially as previously described (Schwinn et al., 1991). The reaction (50 μl final volume) consisted of membranes (~10 μg) and 2.8 mM phosphoenolpyruvate, 0.06 mM GTP, 0.12 mM ATP, 0.1 mM cAMP, 4 U/ml myokinase, 10 U/ml pyruvate kinase, 0.1 mM ascorbic acid, and 3 × 10⁶ dpm [α-³²P]ATP. Typically, reactions contained various concentrations of isoproterenol, 10 mM NaF, or 100 μM forskolin and were carried out for 10 min at 37°C. To estimate the contribution of β₁AR to the total βAR stimulation, other experiments were carried out by incubating membranes at 37°C for 5 min with 1.0 μM ICI118551 (a relatively selective β₂AR antagonist) to which isoproterenol was added (final concentration 10 μM) and the incubations continued for 10 min. β₂AR-mediated stimulation was estimated by carrying out reactions with the relatively selective partial β₂AR agonist zinterol (1.0 μM) under the same conditions. In some studies, myocytes were isolated from the hearts as described (Masaki et al., 1997), and membranes were prepared. Adenylyl cyclase activities were then determined with these membranes as outlined above. Reactions were stopped by dilution with 1.0 ml of a 4°C solution containing excess ATP and cAMP, and 25,000 dpm/ml [³H]cAMP used for column recovery. [³²P]cAMP was separated by chromatography over alumina columns (Alvarez and Daniels, 1990).

¹²⁵I-Cyanopindolol (CYP) Binding. For agonist competition studies (Green and Liggett, 1994), membranes were prepared as above except that two additional 40,000g centrifugations were carried out and the membranes were resuspended in a buffer providing for 50 mM HEPES, 5 mM MgCl₂, pH 7.40 in the final reaction. Incubations were carried out with 40 pM ¹²⁵I-CYP with varying concentrations of isoproterenol with 0.1 mM ascorbic acid for 1 h at 37°C. For determination of total receptor density, reactions were carried out with membranes from the adenylyl cyclase preparation (see above) using 400 pM ¹²⁵I-CYP in the absence and presence of 1.0 μM alprenolol, used to define nonspecific binding. Binding reactions were terminated by dilution and rapid filtration over GF/C (Whatman, Tewksbury, MA) filters.

[³H]Forskolin Binding. [³H]forskolin binding was carried out by methods similar to those described by others in rat heart (Shu and Scarpace, 1994). Ventricles were homogenized as above in a 4° buffer consisting of 250 mM sucrose, 1 mM MgCl₂, 5 mM Tris, pH 7.40, and 5 μg/ml of leupeptin, benzamidine, and soybean trypsin inhibitor and

then centrifuged at 40,000g for 10 min. The pellet was resuspended in buffer containing 8 mM MgCl₂, 50 mM HEPES, pH 7.4, and the above protease inhibitors. Reactions consisted of membranes (~250 μg protein), 40 nM [³H]forskolin, and varying concentrations of unlabeled forskolin, and were carried out for 1 h at 25°C and terminated by dilution and filtration over GF/C filters.

Kinase Expression/Function. Phospholipid-stimulated incorporation of ³²P into PHAS-I (D'Angelo et al., 1997) was used as an assay of total protein kinase C (PKC) activity in mouse hearts using components from Stratagene (La Jolla, CA) and Amersham (Arlington Heights, IL). Briefly, mouse hearts frozen at -80°C were thawed, homogenized, and separated into cytosolic and membrane fractions by centrifugation at 100,000g for 30 min. Then, 50 μg of each fraction was assayed for PKC activity by coincubation for 10 min at 30°C with 0.1 mM ATP plus 1 μCi [γ-³²P]ATP and 0.5 μg/μl PHAS-I in the presence and absence of phospholipid and CaCl₂. Purified rat brain PKC was included as a positive control, and PHAS-I was omitted for a negative control. Phosphorylated proteins were resolved on 10% SDS-polyacrylamide gel electrophoresis (PAGE) gels and phosphorylation of the 21-kDa PHAS-I protein quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Results are shown for whole homogenate (total PKC content) or membrane versus cytosolic activity (endogenous activation). PKA activity was assessed essentially as previously described (McGraw et al., 1998). Briefly, cytosolic fractions were incubated in a reaction mixture containing 20 mM 4-morpholinepropanesulfonic acid, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium vanadate, 15 mM MgCl₂, 10 μM PKC inhibitor (PKC 19-36; Life Technologies, Rockville, MD), 125 μM [γ-³²P]ATP (~4,000 cpm/pmol), and 125 μM kemptide for 10 min at 30°C. Other reactions included cAMP, thus providing for maximal stimutable protein kinase A (PKA) activity. The reactions were stopped by spotting the assay mixture onto P81 phosphocellulose paper. The filters were washed two times with 1% phosphoric acid and once with water. Bound radioactivity was measured by liquid scintillation counting. βAR kinase (BARK) expression in heart homogenates was determined by Western blots as described previously (McGraw and Liggett, 1997).

Western Blots. Western blots were carried out essentially as previously described (D'Angelo et al., 1997; McGraw and Liggett, 1997; Jewell-Motz et al., 1998) with polyclonal antisera (Santa Cruz Biotechnology, Santa Cruz, CA) at titers of 1:1000 for G_{αs}, G_{α12}, G_{α13}, G_{αq/11}, G_{α12}, and GRK2 (BARK1) and 1:200 for type V/VI adenylyl cyclase. For PKC studies, protein expression of isoforms was measured by Western immunoblot techniques using direct fluorescence detection with Cy⁵-linked secondary antibody or enhanced chemifluorescence. Ventricular homogenates, or cytosolic (100,00g supernatant) and Triton X-100 extracted particulates (100,00g pellet), were size-separated on 8% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat dry milk and incubated with primary PKC isoform-specific antibody (Santa Cruz Biotechnology) for 1 h followed by appropriate secondary antibody. The blots were developed with enhanced chemifluorescence or directly detected for fluorescence using a STORM imaging system (Molecular Dynamics). In each series of experiments, studies were performed using purified recombinant human PKC isoforms to generate standard curves. Different amounts of ventricular protein were used to optimize protein contents and ensure that signals generated were within the standard curve. PKC contents are expressed in nanograms of PKC per milligram of tissue protein. Protein loading and efficacy of transfer to polyvinylidene difluoride membranes was evaluated by amino black staining of the membrane after immunoblotting.

Analysis of PKC Isoform mRNA. PKCα and ε mRNA were analyzed using a modification of a previously described PCR-based method that permits the simultaneous amplification of multiple PKC isoforms using degenerate oligonucleotide primers complementary to conserved sequences in cysteine-rich and ATP-binding regions of the conventional and novel PKCs (Kohout and Rogers, 1993; Ali et al.,

1994). Individual products were distinguished by hybridization with ^{32}P -labeled isoform-specific oligodeoxynucleotide probes. Total RNA was reverse-transcribed using oligo(dT) templates. PCR was performed as described (Ali et al., 1994), and aliquots were removed at increasing cycle numbers as indicated. After size-separation on 1% agarose gels and blotting onto nylon membranes, PCR products were quantitated from PKC isoform-specific Southern blots (Ali et al., 1994) using a PhosphorImager and plotted as a function of cycle number. β -actin PCRs were run simultaneously to control for loading and cDNA integrity. Northern blot analysis of poly (A)⁺ mRNA was performed using standard techniques with the cDNAs for rat PKC α and PKC ϵ as radiolabeled probes.

Patch-Clamp Studies. Single ventricular myocytes were isolated from the hearts of nontransgenic (NTG) and $G_{\alpha q}$ mice, and whole-cell currents were recorded using patch-clamp techniques as previously described (Masaki et al., 1997). Briefly, the heart was perfused with Ca^{2+} -free Tyrode's solution containing collagenase type I (Worthington; 0.5 mg/ml) and BSA (1 mg/ml) for 30 to 40 min by the Langendorf method at 37°C. At the end of the perfusion period, the heart was removed and left ventricular tissues sieved through 200- μm nylon mesh and centrifuged for 2 min at 1000g. Isolated cardiomyocytes were stored in low Cl^- , high K^+ medium and all experiments were performed at 20 to 22°C. The patch pipettes had a resistance of 2 M Ω or less. The experimental chamber (0.2 ml) was placed on a microscope stage, and the external solution changes were made rapidly using a modified Y-tube technique (Yamamoto et al., 1996). The external solution contained 2 mM CaCl_2 , 1 mM MgCl_2 , 135 mM tetraethyl ammonium chloride, 5 mM 4-aminopyridine, 10 mM glucose, 10 mM HEPES, pH 7.3. The pipette solution consisted of: 100 mM cesium aspartate, 20 mM CsCl, 1 mM MgCl_2 , 2 mM ATP, 0.5 mM GTP, 10 mM BAPTA, and 5 mM HEPES, pH 7.3. These external and internal solutions provided isolation of Ca^{2+} channel currents (I_{Ca}) from other membrane currents such as Na^+ and K^+ channel currents and also Ca^{2+} flux through the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Wier, 1990). Data are presented as mean \pm S.E. of n number of myocytes studied, which were derived from three to five mice.

Statistical Analysis. Results from studies were compared by paired or unpaired t -tests as appropriate with $P < .05$ considered significant. Curve fitting was carried out using a nonlinear, iterative, least-squares method with PRISM software (Graphpad, San Diego, CA). Except as noted, data are presented as mean \pm S.E. of the indicated number of independent experiments, each performed with a different mouse.

Results

Functional coupling of βAR was assessed in membrane adenylyl cyclase and whole-cell patch-clamp studies. As shown in Fig. 1A, isoproterenol-stimulated activities were markedly decreased in cardiac membranes derived from the $G_{\alpha q}$ 40 transgenic mice compared with age-matched NTG littermates. Maximal stimulation was 19.7 ± 2.6 pmol/min/mg in the $G_{\alpha q}$ 40 transgenics compared with 43.7 ± 5.6 in the NTG mice ($P < .005$), with no differences in the K_{act} ($pK_a = 6.48 \pm 0.07$ versus 6.61 ± 0.15). Additional adenylyl cyclase studies were carried out with another $G_{\alpha q}$ transgenic line denoted $G_{\alpha q}$ 25 (D'Angelo et al., 1997). As shown in Fig. 1A, these mice had depressed βAR function very similar in magnitude to that of the $G_{\alpha q}$ 40 mice (isoproterenol-stimulated activity = 14.8 ± 5.51 pmol/min/mg). As shown in Fig. 1B, basal (nonagonist stimulated) adenylyl cyclase activities were also lower in both lines of $G_{\alpha q}$ mice, as were those stimulated by NaF and forskolin. Given that these phenotypes were the same, subsequent studies were confined to the $G_{\alpha q}$ 40 line (heretofore referred to as $G_{\alpha q}$ transgenics). Be-

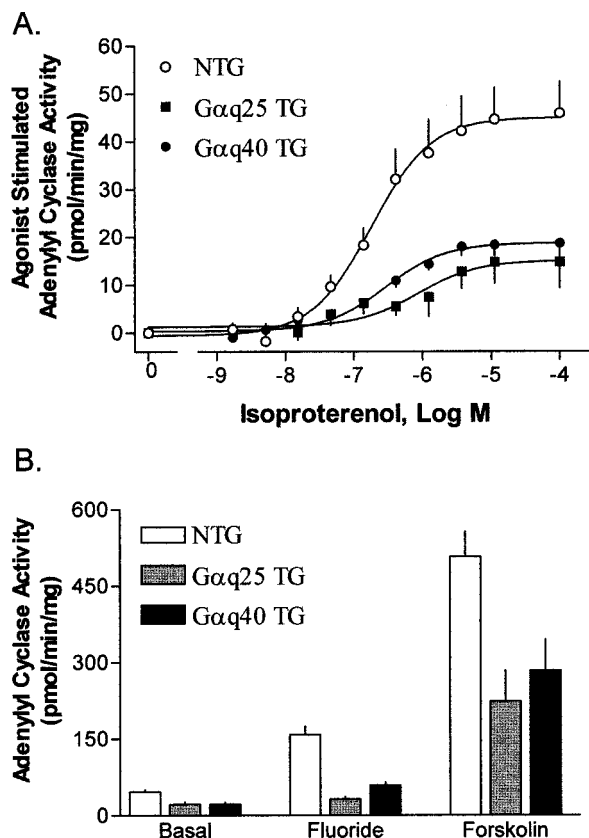


Fig. 1. Cardiac adenylyl cyclase activity is impaired in $G_{\alpha q}$ transgenic mice. A, results from isoproterenol dose-response studies (basal levels subtracted). B, basal, 10 mM NaF, and 100 μM forskolin responses. All responses from $G_{\alpha q}$ transgenic mice shown in A and B were significantly ($P < .01$) less than those of NTG littermates. Data are from five independent experiments carried out with five mice in each group.

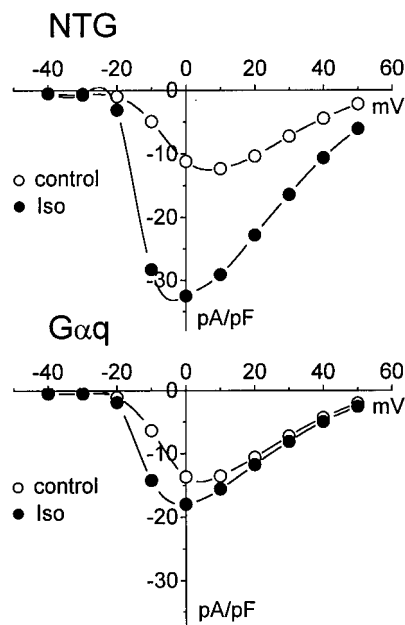


Fig. 2. Isoproterenol-stimulated I_{Ca} is reduced in isolated myocytes from $G_{\alpha q}$ transgenic mice. Shown is a representative whole-cell patch-clamp study. Maximal isoproterenol-stimulated increases in I_{Ca} were $124 \pm 13\%$ ($n = 19$) over baseline in NTG myocytes, compared with $30 \pm 5\%$ ($n = 23$) over baseline in the $G_{\alpha q}$ transgenic myocytes ($P < .001$). Baseline I_{Ca} density normalized to myocyte size measured by cell capacitance was not different between the lines.

cause an important consequence of βAR activation in the heart is the phosphorylation of L-type Ca²⁺ channels, single-cell patch-clamp studies were also undertaken in myocytes from G_{αq} and NTG mice (Fig. 2). Baseline I_{Ca} was the same in myocytes from the two lines. Maximal isoproterenol-stimulated increases in I_{Ca} were 124 ± 13% (n = 19) over baseline in NTG myocytes. In contrast, the maximal agonist-promoted increases in I_{Ca} in the G_{αq} mice were only 30 ± 5% (n = 23) over baseline, representing an ~75% impairment compared with NTG myocytes (P < .001). To confirm that the defect found in crude cardiac membranes was indicative of an alteration in myocyte receptor-adenylyl cyclase signaling, membranes were prepared from isolated myocytes and activities determined. As shown in Fig. 3, absolute basal and isoproterenol-stimulated activities were significantly depressed in myocyte membranes from the G_{αq} mice. Furthermore, the isoproterenol fold-stimulation over basal was ~50% less (2.29 ± 0.51 versus 4.03 ± 0.71-fold, n = 4, P < .01) in these myocytes compared with those of NTG littermates.

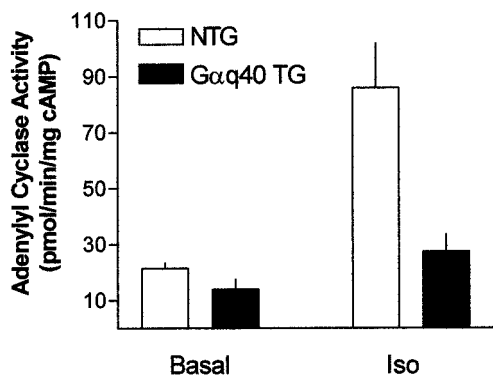


Fig. 3. βAR-stimulated adenylyl cyclase activity is decreased in myocytes from G_{αq} transgenic hearts. Intact myocytes were isolated, membranes prepared, and adenylyl cyclase activities determined in the presence of water (basal) or 10 μM isoproterenol (ISO). The absolute levels of basal and isoproterenol-stimulated adenylyl cyclase were decreased in the G_{αq} transgenic myocytes (P < .01) as were the isoproterenol fold-stimulations over basal (P < .01). Results are from four experiments.

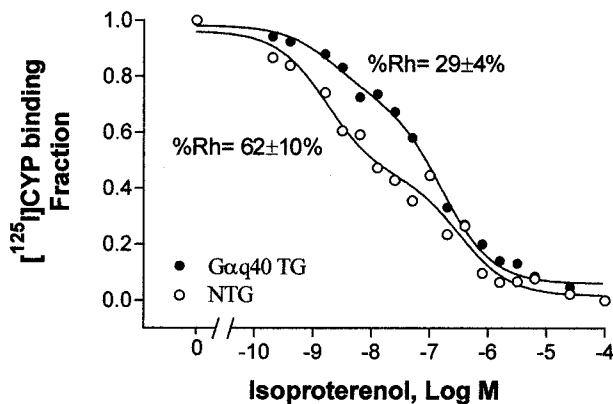


Fig. 4. Decreased agonist-promoted accumulation of the receptor high-affinity complex in membranes from G_{αq} transgenic mice. Agonist competition studies were carried out in the absence of guanine nucleotide as described in *Materials and Methods*. Shown are mean data from five independent studies from five mice in each group. Error bars are omitted for clarity. The percentage of receptors in the high-affinity state (%R_H) was calculated from each individual curve and is decreased in the G_{αq} mice (P < .02) as shown. Other parameters (pK_H = 8.94 ± 0.10 versus 8.98 ± 0.28, and pK_T = 7.07 ± 0.05 versus 6.31 ± 0.43) were not different between G_{αq} and NTG littermates.

To assess βAR subtype-specific coupling, adenylyl cyclase activities were determined with the relatively β₂AR-selective partial agonist zinterol, and with isoproterenol in the presence of the relatively selective β₂AR antagonist ICI118551 for an indication of β₁AR coupling (see *Materials and Methods*). Although this approach does not provide for absolute selective activation of one or the other subtype, comparisons between G_{αq} and NTG mice do allow for a relative determination of potential differences in signaling under identical conditions. In four such experiments, the zinterol response over basal was 11.3 ± 3.6 versus 6.4 ± 1.5 pmol/min/mg for NTG compared with G_{αq} mice, equivalent to an ~40% desensitization of β₂AR. β₁AR stimulation, assessed as described, was 10.1 ± 2.3 pmol/min/mg over basal with NTG mice, compared with only 2.0 ± 1.0 pmol/min/mg with the G_{αq} mice (P < .02). Thus, β₁AR function was impaired ~80% in the G_{αq} mice.

We considered that enhanced activity of kinases known to phosphorylate and uncouple βAR (GRKs, PKA, PKC) was a potential mechanism for the βAR dysfunction observed in the G_{αq} mice. βARK levels were determined by Western blots and were not found to be increased, but in fact decreased in the G_{αq} mice (Fig. 5). In contrast, intrinsic PKA activity was not found to be altered in the G_{αq} mice, nor did total stimutable in vitro PKA activity differ between the two sets of mice (Fig. 6A). Increased PKC activity was considered a probable candidate because overexpression of G_{αq} could result in sustained diacylglycerol-mediated stimulation of certain PKC isoenzymes, and because we have previously shown translocation of cardiac PKCε in these mice (D'Angelo et al., 1997). Figure 6B shows that the levels of total PKC, measured as phosphorylation of PHAS-1 protein by whole-heart

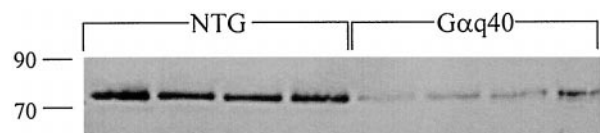


Fig. 5. Cardiac βARK expression is decreased in G_{αq} transgenic mice. Shown are the results of Western blots with cardiac lysates from four NTG and four G_{αq} transgenic mice.

homogenates, are increased in the $G_{\alpha q}$ mice 2.6 ± 0.8 -fold ($n = 4$) over NTG mice. The ratio of PKC in membrane particulates compared with cytosol, which is a measure of the activation state of PKC, was maintained between the two groups of mice. Thus, because overall PKC levels are increased in the $G_{\alpha q}$ mice over NTG levels, the absolute levels of activated PKC are elevated in these mice. To ascertain which PKC isoforms undergo changes in expression, PKC isoform content was assayed by quantitative immunoblotting. The most abundant PKC isoform was PKC α , which was expressed at levels approximately five times that of PKC ϵ (Fig. 7A). Ventricular PKC α in $G_{\alpha q}$ overexpressors was up-regulated by 76% (NTG 712 ± 114 versus $G_{\alpha q}$ 1255 ± 158 ng/mg protein, $n = 4$ pairs, $P < .01$), whereas PKC ϵ was down-regulated in $G_{\alpha q}$ mice by 26% (NTG 158 ± 10 and $G_{\alpha q}$ 117 ± 12 ng/mg protein, $n = 8$ pairs, $P < .02$). Levels of PKC δ and η did not differ (not shown). Intrinsic activation of PKC α and ϵ isoforms was assessed by their relative particulate and soluble partitioning. Consistent with the results of the phosphorylation studies, the absolute amount of particulate-associated PKC α in $G_{\alpha q}$ mice was greater than NTG, indicating an increase in the amount of activated PKC α in $G_{\alpha q}$ mice,

although the ratio of particulate to cytosol PKC α was not altered in the $G_{\alpha q}$ overexpressors (NTG 0.51 ± 0.13 versus $G_{\alpha q}$ 0.47 ± 0.14 , $n = 4$, $P = \text{NS}$). Confirming our previous report (D'Angelo et al., 1997), PKC ϵ translocation, measured as relative particulate fractioning, was significantly increased (NTG 1.17 ± 0.09 versus $G_{\alpha q}$ 2.38 ± 0.18 , $n = 8$ pairs, $P < .001$).

Studies using RT-PCR and Northern blots of PKC isoform mRNA from NTG and $G_{\alpha q}$ mouse hearts indicate more abundant PKC α mRNA by ~ 2 -fold in the $G_{\alpha q}$ overexpressors (Fig. 7, B and C), consistent with the observed increased PKC α protein. In contrast, $G_{\alpha q}$ PKC ϵ mRNA levels are identical with controls. Thus, these studies suggest that up-regulation of PKC α in $G_{\alpha q}$ overexpressors may be transcriptionally mediated, but that down-regulation of PKC ϵ is post-transcriptional.

We also considered that the expression of G_s could be decreased or that of G_i increased in these mice relative to NTG. Because NaF activates both of these G-proteins, such changes would be consistent with the depressed NaF-stimulated adenylyl cyclase activities. Shown in Fig. 8 are the results of Western blots from four mice in each group. For these experiments, $G_{\alpha 12}$, which was not expected to change, acted as a control. As can be seen, both $G_{i\alpha 2}$ and $G_{i\alpha 3}$ levels were increased in the $G_{\alpha q}$ overexpressing mice, whereas G_s levels were unchanged. To assess the potential contribution of the increase in G_i to the phenotype, mice were treated in vivo with pertussis toxin (100 $\mu\text{g/kg}$), which dissociates receptor- G_i interaction by ADP-ribosylation of the α_i -subunit. However, three of the four $G_{\alpha q}$ mice so treated died within 12 h, whereas none of the NTG littermates treated in the same manner showed any untoward effects. Because in vivo pertussis toxin treatment had lethal effects in the $G_{\alpha q}$ mice, which precluded studying membrane adenylyl cyclase activities, isolated intact myocytes from the two groups were treated with 5 $\mu\text{g/ml}$ pertussis toxin for 6 h, membranes prepared, and adenylyl cyclase activities measured as before. Results of these experiments are shown in Fig. 9. In NTG mice, the toxin increased both basal and isoproterenol-stimulated activities, but the isoproterenol fold-stimulation over basal was not altered. In membranes from $G_{\alpha q}$ myocytes, pertussis toxin increased the isoproterenol fold-stimulation from 2.29 ± 0.51 to 3.38 ± 0.23 ($P < .05$). However, this fold-stimulation after toxin was not of the magnitude found with untreated NTG myocytes (4.03 ± 0.71 -fold), consistent with there being defects other than that evoked by the increase in G_i . Interestingly, responses to NaF and forskolin were not enhanced by pertussis toxin in myocytes from NTG or $G_{\alpha q}$ mice. These results suggest that the increase in G_i has a contribution to the βAR signaling defect, but not the responses to NaF or forskolin, in the $G_{\alpha q}$ mouse. We wondered whether an increase in G_i might also account for the decreased $\%R_H$ observed in agonist-competition studies with these mice. Pertussis toxin caused significant increases in nonspecific ^{125}I -CYP binding and poor replicates in these assays with myocytes, so the effect of increased G_i could not be directly assessed using the toxin. Because we also wanted to assess the effects of increased G_i in isolation on high-affinity receptor binding (i.e., in the absence of changes in other signal transduction elements), we used human embryonic kidney (HEK) 293 cells transfected to express $\beta_2\text{AR}$, or $\beta_2\text{AR}$ and $G_{i\alpha 2}$, as a model system to explore these issues.

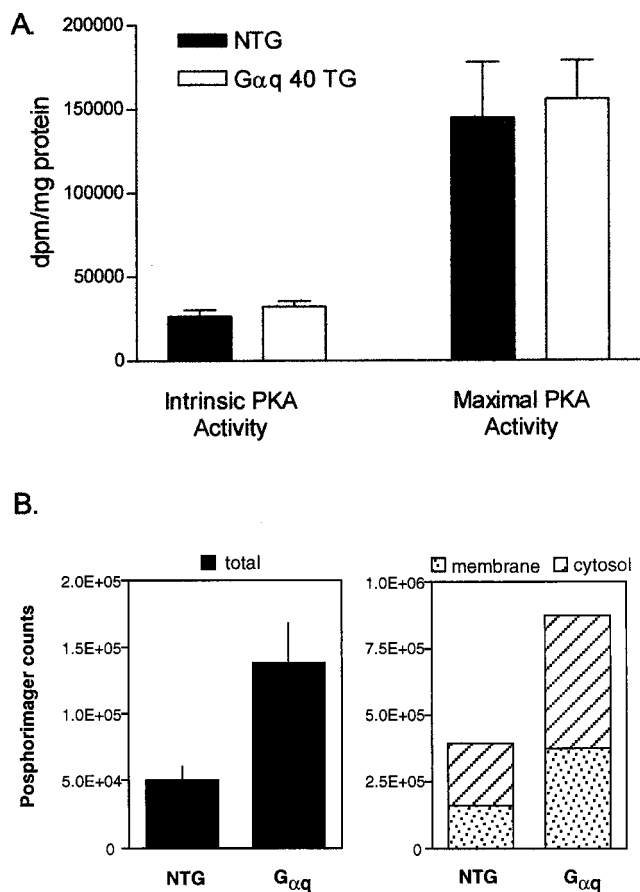


Fig. 6. Cardiac PKC, but not PKA, is increased in $G_{\alpha q}$ transgenic mice. In A, homogenates from the two lines were used to phosphorylate a synthetic PKA substrate in the absence (intrinsic) and presence (maximal) of cAMP. No differences were observed between $G_{\alpha q}$ and NTG littermates. In B, phosphorylation of a synthetic PKC peptide substrate by homogenate, cytosolic, and membrane fractions from hearts are shown. The total PKC activity is increased in the $G_{\alpha q}$ mice, and the ratio of cytosolic to membrane fractions is maintained. Shown are results of four to six independent experiments. * $P < .01$.

The amount of the G_{iα2} construct used in the transfections was adjusted so that overexpression of ~5-fold was attained to mimic what was observed in the G_{αq} hearts (see inset to Fig. 10). Agonist competition binding parameters and adenylyl cyclase activities were then determined in membranes from the two sets of cells. Membranes from cells expressing the increased G_i displayed no significant decrease in the percentage of receptors in the high-affinity state (%R_H = 14 ± 1.5 versus 18 ± 6.8, *n* = 4, *P* > .05) and no change in binding affinities. Adenylyl cyclase studies revealed small decreases in basal (from 8.0 ± 0.7 to 5.8 ± 1.1 pmol/min/mg, *P* < .05) and isoproterenol (from 23.7 ± 2.2 to 17.9 ± 1.8 pmol/min/mg)-stimulated activities due to increased G_{iα2} (Fig. 10). There were no significant changes in the response to NaF (14.7 ± 0.1 to 12.9 ± 1.9 pmol/min/mg, *P* > .05) or forskolin (75.3 ± 6.6 to 60.4 ± 7.5 pmol/min/mg, *P* > .05) induced by G_i overexpression. Taken together with the pertussis toxin experiments with myocytes, the data are consistent with the increase in G_i having a small contribution to

the dysfunctional βAR signaling phenotype, but not the decreased responsiveness to NaF and forskolin.

The decreased basal, NaF, and forskolin-stimulated adenylyl cyclase enzymatic activities implicated a potential decrease in expression of cardiac adenylyl cyclases in the G_{αq} mice. Western blots with a type V/VI antisera revealed a dominant signal at the molecular mass for type V adenylyl cyclase, consistent with other studies (Yu et al., 1995) indicating that type V is the major cardiomyocyte adenylyl cyclase isoform. G_{αq} mice displayed a decrease on the order of ~50% of the adenylyl cyclase type V isoform (Fig. 11A). However, the signals were somewhat weak, as previously reported (Ping et al., 1997). To provide a greater degree of quantitation, [³H]forskolin binding experiments were carried out. In rat heart, a high correlation has been found between adenylyl cyclase protein expression and [³H]forskolin binding (Shu and Scarpace, 1994). Such binding (Fig. 11B) amounted to 133 ± 8 fmol/mg in NTG mice compared with 72 ± 11 fmol/mg in G_{αq} mice (*n* = 4, *P* < .01). This ~46%

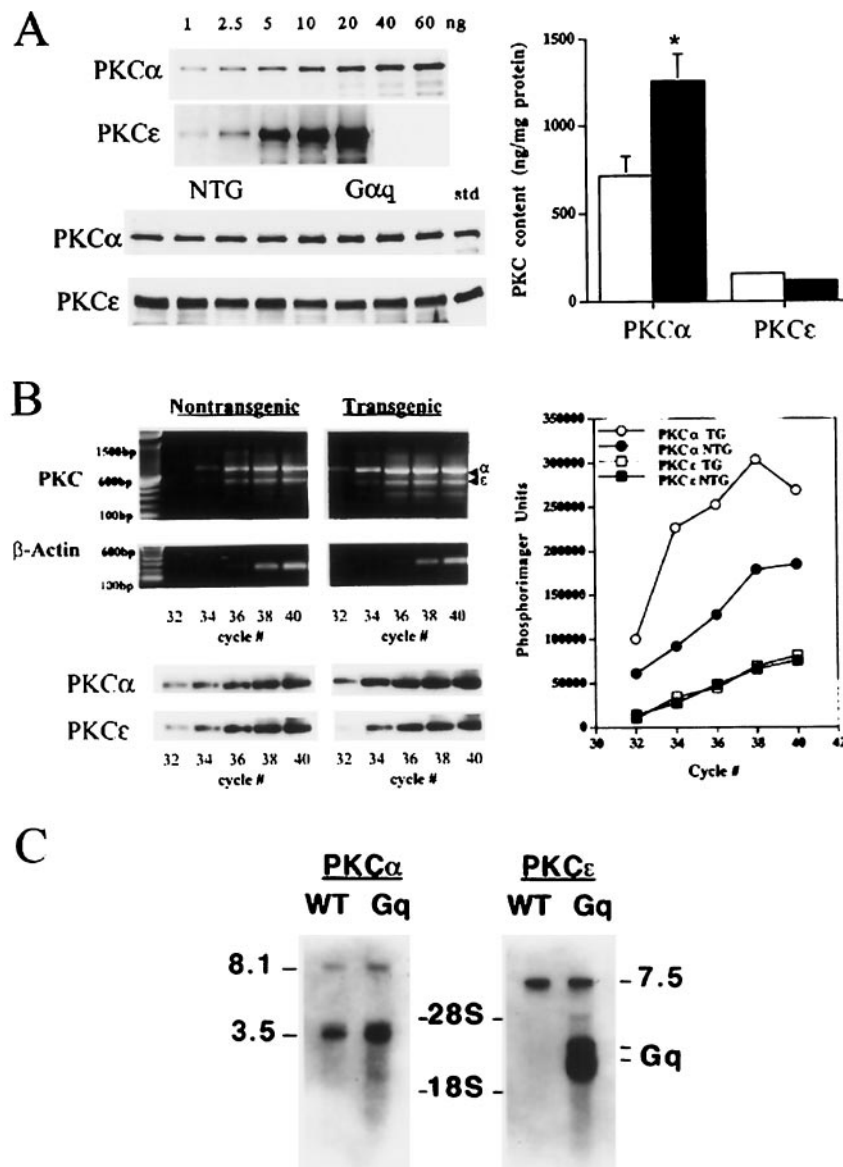


Fig. 7. PKC isoform protein and mRNA content in G_{αq} transgenic hearts. **A**, PKC protein content was assessed by quantitative immunoblot analysis. The assay was linear in the range of 1 to 60 ng for purified PKCα and 1 to 20 ng for purified PKCε (top). Ventricular homogenates were size separated by SDS-PAGE and immunoblotted (2.5-fold greater protein was loaded for PKCε compared with PKCα). Cumulative results from four to eight individual hearts are on right. **P* < .01 versus NTG. **B**, analysis of PKC isoform mRNA expression by RT-PCR. Left, ethidium-stained gel of PCR products and resulting Southern blot using isoform-specific probes. Right, results from a single experiment representative of three performed showing a higher expression of PKCα, but not PKCε, in the G_{αq} transgenic hearts. **C**, Northern analysis of poly A⁺ RNA (8 μg/lane) shows increased 8.1- and 3.5-kb PKCα transcripts, but no change in 7.5-kb PKCε transcript. The PKCε Northern analysis was simultaneously probed for G_{αq} as indicated. Shown is a representative result from four experiments.

decrease in expression is similar in magnitude to the decreases in basal and forskolin-stimulated activities and the decrease in type V adenylyl cyclase protein expression observed.

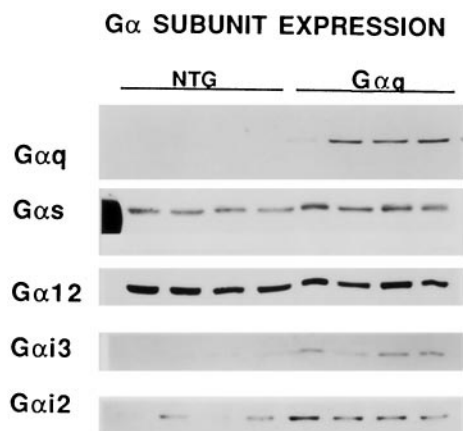


Fig. 8. G_{α} subunit expression in NTG and $G_{\alpha q}$ transgenic hearts. Shown are results of Western blots from four hearts in each group. Signals from $G_{\alpha 12}$ blotting acted as a control for loading and transfer, whereas those from $G_{\alpha q}$ confirmed transgene overexpression. $G_{\alpha i3}$ and $G_{\alpha i2}$, but not $G_{\alpha s}$, were increased in the $G_{\alpha q}$ transgenic hearts.

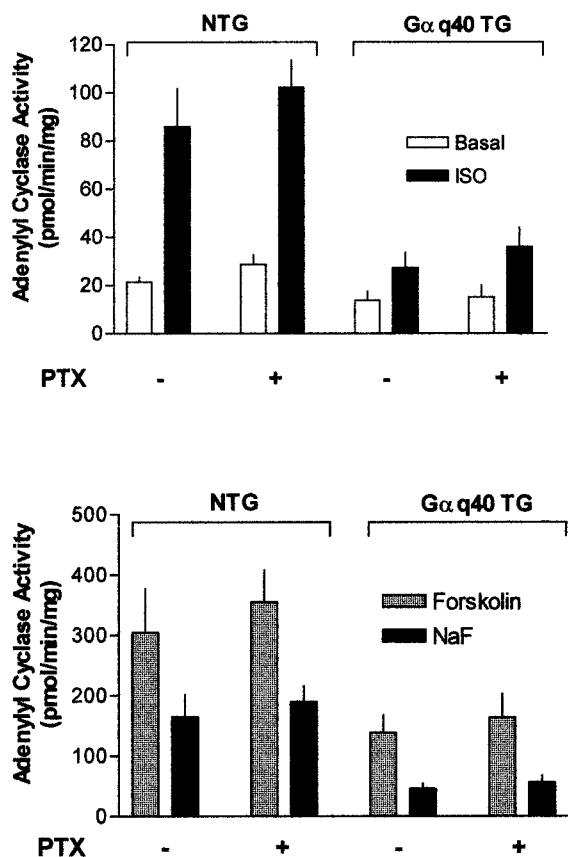


Fig. 9. Pertussis toxin causes an increase in isoproterenol-stimulated adenylyl cyclase activity in myocytes from $G_{\alpha q}$ transgenic mice. Intact myocytes were isolated and treated with 5 μ g/ml pertussis toxin or vehicle alone for 6 h, membranes were prepared, and adenylyl cyclase activities were determined in the presence of water (basal), 10 μ M isoproterenol (ISO), 10 mM NaF, or 100 μ M forskolin. Shown are results from four experiments. See Results for interpretation.

Discussion

A number of cell and animal models of hypertrophy have pointed toward common pathways that can be initiated by G_q signaling (Dorn and Brown, 1999). In rat neonatal myocytes, exposure of cells to agonists for the G_q -coupled α_1 -adrenergic, endothelin, angiotensin-II, and prostaglandin $F_{2\alpha}$ receptors results in a hypertrophic response (Shubeita et al., 1990; Knowlton et al., 1993; Sadoshima et al., 1993; Adams et al., 1996). Aortic banding in guinea pigs, which causes pressure overload hypertrophy, results in a temporally related increase in PKC_{ϵ} (Paul et al., 1997), and transgenic overexpression of a constitutively activated α_{1B} AR results in mild hypertrophy (Milano et al., 1994). In addition, transgenic expression of an inhibitor of G_q function (G_q minigene; Akhter et al., 1998) renders mice resistant to aortic banding-

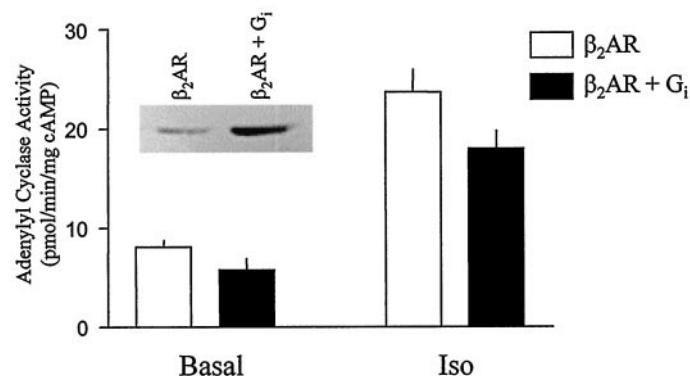


Fig. 10. Increased $G_{\alpha i2}$ causes a decrease in basal and isoproterenol-stimulated adenylyl cyclase activity in HEK293 cells. HEK293 cells were transfected to express β_2 AR (~300 fmol/mg) or the same levels of β_2 AR plus $G_{\alpha i2}$ (~5-fold over endogenous levels, see inset). Shown are results from four experiments, where basal and isoproterenol-stimulated levels were lower ($P < .02$) when $G_{\alpha i2}$ was overexpressed. NaF and forskolin-stimulated levels were not changed by $G_{\alpha i2}$ overexpression.

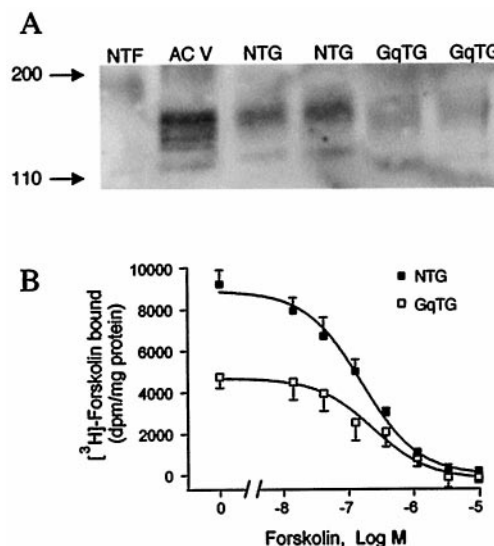


Fig. 11. Adenylyl cyclase expression is decreased in $G_{\alpha q}$ transgenic hearts. A, Western blots show a decrease in type V adenylyl cyclase (AC V). B, in membranes from $G_{\alpha q}$ transgenic mice, [³H]forskolin binding, used to quantitate changes in cardiac adenylyl cyclase expression, was 46% less in the $G_{\alpha q}$ transgenic mice compared with NTG littermates (72 ± 11 versus 133 ± 8 fmol/mg, respectively, $P < .01$). Shown are results from independent experiments performed with five hearts from each group.

induced cardiac hypertrophy. To explore the basis of the above findings, we have recently created transgenic mice overexpressing the α-subunit of G_q (D'Angelo et al., 1997). Such expression initiates signaling at an early point in the cascade, thus allowing for a hierarchical assessment of the multiple potential events that may mediate development of the ultimate cardiac phenotype. Because these events occur in the absence of the systemic effects of continuous agonist infusions or hemodynamic loading, this approach provides for a cardiac phenotype that results purely from altered biochemical signaling events. The phenotype of G_{αq} overexpression includes myocyte hypertrophy, increased cardiac mass, and increased left ventricular chamber dimensions consistent with an eccentric form of hypertrophy. In addition, a program of fetal gene expression, observed in many other forms of experimental hypertrophy, is recapitulated in G_{αq} mice (D'Angelo et al., 1997). Ventricular function, as assessed by echocardiography and invasive hemodynamic measurements, is depressed at rest, and the response to infused β agonist is markedly impaired (D'Angelo et al., 1997). This nearly absent response to β agonist stimulation occurs without a loss of cardiac βAR expression, thus providing an opportunity to assess regulatory mechanisms evoked by G_q signaling that are distinct from receptor down-regulation.

In the G_{αq} mice, a significant impairment of isoproterenol-stimulated adenylyl cyclase activity was observed, which appears to be due to dysfunction of both β₁- and β₂AR subtypes. To assess the consequences of such decreased βAR function within the context of a physiologically relevant signaling event in the cardiomyocyte, whole-cell patch-clamp studies were undertaken. βAR-mediated increases in Ca²⁺ influx via the opening of L-type Ca²⁺ channels were markedly depressed in the G_{αq} mice. The extent of βAR desensitization was comparable with the two measurements (~56% with cardiac membrane adenylyl cyclase assays and ~75% with patch-clamp studies). Isolated myocyte membrane adenylyl cyclase studies revealed a similar impairment. Three mechanisms were identified that likely together result in this βAR signaling defect. First, direct βAR coupling to G_s appears to be impaired. This conclusion is based on the loss of agonist high-affinity binding sites in the G_{αq} mice as determined in agonist competition studies carried out in the absence of guanine nucleotide. The decrease in the fold-stimulation of adenylyl cyclase by isoproterenol in the myocyte studies, which is independent of the absolute levels of activity, is also consistent with a receptor defect. Such uncoupling could occur when βAR are phosphorylated by GRKs, PKA, or PKC. A decrease in %R_H might also be due to a decrease in G_{αs} or an increase in G_{αi}, although G-proteins are thought to be in excess in relation to βAR expression in the heart. Nevertheless, a decrease in G_{αs} was not observed. However, an increase in G_{αi} was in fact found. In the model HEK293 cell system used to explore whether this could cause a decrease in agonist high-affinity binding, no change in %R_H was observed when G_{αi2} was overexpressed ~5-fold. However, basal and agonist-stimulated adenylyl cyclase activities in these cells were decreased with G_{αi2} overexpression. Taken together with the pertussis toxin studies (see below), it is apparent that the increase in G_{αi} observed in the G_{αq} mice account for some of the βAR uncoupling observed.

The levels of βARK, the predominant GRK in the heart, were found to be depressed in G_{αq} mice. This is in contrast to

the muscle lim protein knockout mice, which exhibit hypertrophy, decreased agonist responsiveness, and increased levels of βARK (Rockman et al., 1998). The level of intrinsic activity of PKA, and the in vitro maximal stimutable levels of activity, were not different between G_{αq} and NTG mice. In contrast to the above, the absolute levels of activated PKC were found to be clearly increased in the G_{αq} mice by ~2.5-fold. As shown, this enhancement is due to an increase in expression of the most abundant cardiac isoform, PKCα. PKCα mRNA transcripts are also increased by approximately the same extent, indicating a transcriptional component to regulation of this PKC isoform. Because ventricular PKCε content is substantially lower than PKCα, its down-regulation in the G_{αq} transgenic mice (which is likely a consequence of its preferential activation) is offset by the up-regulation of the more abundant α isoform. Thus, an overall enhancement of absolute PKC activity is present in the hearts of the G_{αq} transgenic mice. Although it may at first appear counterintuitive that signaling through a pathway that activates PKC can result in its up-regulation, our findings in this regard are consistent with those of others (Henrich and Simpson, 1988) who found that stimulation of α₁AR of neonatal cardiomyocytes acutely activated and chronically up-regulated total PKC activity. In considering, then, which kinase is responsible for the observed βAR dysfunction, PKC phosphorylation appears to be the most likely mechanism for this receptor-G_s uncoupling. Indeed, PKC has been shown to phosphorylate β₁AR and β₂AR in vitro and in intact cells, leading to functional desensitization (Bouvier et al., 1987, 1991; Freedman et al., 1995). Although phosphorylation by βARK is also a potential candidate mechanism, the fact that its expression is decreased makes it less likely. Also, we have recently created double transgenic mice expressing G_{αq} and a βARK inhibitor (Dorn et al., 1999). These mice showed no improvement in ventricular function compared with G_{αq} littermates. Thus, we conclude that βARK-mediated phosphorylation of βAR is not a major mechanism of receptor dysfunction in the G_{αq} mice. Finally, PKA phosphorylation appears to be an unlikely candidate, given that its activity is low in the G_{αq} mice and is equivalent to NTG littermates. Presumably, the extensive desensitization of βAR signaling (and lower basal levels of adenylyl cyclase activity) have limited the effectiveness of this cAMP-dependent mechanism (McGraw et al., 1998) despite probable elevated levels of catecholamines in the G_{αq} mice.

At the level of G protein expression, we found that G_{αi2} and G_{αi3} levels are increased in transgenic mice, whereas G_s levels are unchanged. Similar findings have been reported in human heart failure (Feldman et al., 1988; Eschenhagen et al., 1992). Such an increase in G_i could act to lower basal levels of adenylyl cyclase activity through inhibition of the enzyme. In addition, β₂AR have recently been shown to couple to G_i but only when the receptors are phosphorylated by PKA (Daaka et al., 1997). In an attempt to block the effects of elevated G_i, animals were treated with pertussis toxin, which resulted in sudden death of G_{αq} but not NTG mice. This suggests that the ablation of G_i function is detrimental in the failing ventricle, because this dose of toxin is well tolerated in NTG littermates. One can hypothesize from these results that the function of G_i-coupled receptors (such as muscarinic or adenosine) may be necessary for compensation in the G_{αq} mice. Alternatively, stimulatory βAR coupling could have

been enhanced after toxin treatment, resulting in increased cardiac energy expenditure in the face of limited metabolic reserves. To test whether β AR signaling was in fact enhanced with such treatment, isolated myocytes were exposed to pertussis toxin. Subsequent membrane adenylyl cyclase studies showed no increase in isoproterenol-stimulated activities over basal due to toxin treatment from NTG membranes and a clear increase from the G_{α_q} membranes. However, neither the absolute activities nor the fold-stimulation from the toxin-treated G_{α_q} myocytes were normalized to NTG values. Additional studies in HEK293 cells showed that high-affinity β_2 AR binding was not affected by overexpression of G_i . Functionally, such overexpression lowered basal and isoproterenol-stimulated, but not NaF or forskolin-stimulated, activities. Thus, we conclude that the increase in G_i has a contribution to the decreased basal and isoproterenol-stimulated adenylyl cyclase activities in cardiomyocytes in transgenic G_{α_q} myocytes.

Finally, we found that the level of type V adenylyl cyclase protein, as determined by Western blots and a [3 H]forskolin binding assay, was depressed by ~46% in the G_{α_q} mice. This level of decrease was similar to the depressed basal and forskolin-stimulated levels of adenylyl cyclase activities. It is interesting to note that in pacing-induced heart failure models, adenylyl cyclase types V and VI mRNAs have been reported to be decreased, as have basal levels of adenylyl cyclase activities (Ishikawa et al., 1994; Ping et al., 1997). And studies with rat neonatal myocytes have suggested that the levels of adenylyl cyclase expression may be limiting factors in the β AR signaling pathway (Post et al., 1995). Taken together with the results of the current study, interventions aimed at increasing an adenylyl cyclase isoform may enhance β AR function in heart failure. Although only speculative, it is attractive to consider that a decrease in adenylyl cyclase expression or function might be due to enhanced PKC activity. The promoter region of type V adenylyl cyclase has not been studied in regard to PKC responsive sites; however, several PKC isoforms are thought to phosphorylate adenylyl cyclases, although the functional consequences are not entirely clear (Kawabe et al., 1994; Lai et al., 1997).

These results point toward several potential targets for drug or genetic intervention that may be effective in heart failure. The feasibility of these maneuvers can be tested by creating additional genetically modified mice within the G_{α_q} background. Recently, we have attempted to overcome the receptor-coupling defect by increasing the overall expression of the β_2 AR subtype (Dorn et al., 1999). Transgenic mice overexpressing by ~30-fold the human β_2 AR in the heart were mated with the G_{α_q} transgenics, and partial phenotypic rescue was obtained. In these mice, adenylyl cyclase activities were not affected but the hypertrophic response, and resting ventricular function were improved. We have recently also mated transgenic mice overexpressing type V adenylyl cyclase with the G_{α_q} mouse to achieve replacement levels of the cyclase. These dual transgenics had a normalization of adenylyl cyclase activities and ventricular function, but hypertrophy persisted (Tepe and Liggett, 1999a). Of note, transgenic mice overexpressing type V adenylyl cyclase alone show no hypertrophy or depressed contractility (Tepe et al., 1999b). These types of studies also verify that the altered β AR- G_s -adenylyl cyclase signaling in this, and likely other, models of cardiac hypertrophy/failure is multifactorial and

that specific defects may be attributed to certain aspects of the phenotype.

In conclusion, we have studied β AR function in a genetic model of cardiac hypertrophy and ventricular dysfunction. The markedly dysfunctional β AR signaling was found to be due to alterations in three elements of the transduction system: an uncoupling of receptor from G_s likely due to receptor phosphorylation by PKC, an increase in $G_{i\alpha}$, and a decrease in adenylyl cyclase expression. Because multiple cell, animal, and human studies have pointed toward the pathways evoked by G_{α_q} signaling as being critical for development of the hypertrophy and/or failure phenotype, our results may be applicable to strategies whereby receptor function could be modulated therapeutically in heart failure. Such interventions at each interface will likely have specific phenotypic consequences.

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