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Transgenic replacement of type V adenylyl cyclase identifies a critical mechanism of β-adrenergic receptor dysfunction in the $G_{\alpha q}$ overexpressing mouse

Nicole M. Tepe, Stephen B. Liggett*

Departments of Pharmacology and Medicine, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Room 7511, Cincinnati, OH 45267, USA

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Abstract Chronic activation of G_q coupled receptors, or overexpression of $G_{\alpha q}$, in cardiomyocytes results in hypertrophy, enhanced expression of fetal genes, decreased basal and β-adrenergic receptor (βAR) stimulated adenylyl cyclase (AC) activities, and depressed cardiac contractility in vivo. Among several abnormalities of the βAR-Gs-AC pathway that occur in $G_{\alpha q}$ overexpressing transgenic mice, we have investigated whether the observed ∼45% decrease in type V AC expression and function compared to non-transgenic (NTG) is the basis of the above phenotype. Transgenic mice were generated that overexpressed by ∼50% the rat type V AC in the heart using the α-myosin heavy chain promoter. These mice were mated with the $G_{\alpha q}$ transgenics resulting in animals (ACV/$G_{\alpha q}$) that had restored levels of forskolin stimulated AC activities in cardiac membranes. In addition, basal cardiac AC activities were normalized in the ACV/$G_{\alpha q}$ mice (NTG = 23 ± 4.4, $G_{\alpha q}$ = 14 ± 3.6, ACV/$G_{\alpha q}$ = 29 ± 5.3 pmol/min/mg) as were maximal isoproterenol stimulated activities (59 ± 8.9, 34 ± 4.6, 52 ± 6.7 pmol/min/mg respectively). Cardiac contractility was also improved by ACV replacement, with increased fractional shortening (51 ± 2%, 36 ± 6%, 46 ± 3% respectively). In contrast, hypertrophy and expression of hypertrophy associated fetal genes were not affected. Thus the observed decrease in type V AC that accompanies the development of the cardiac phenotype in the $G_{\alpha q}$ model is the dominant mechanism of dysfunctional βAR signalling and contractility. In contrast, the decrease in type V AC or βAR signalling to cAMP is not the basis of the hypertrophic response.

Key words: Adenylyl cyclase; cAMP; β-Adrenergic receptor; Hypertrophy; Desensitization

1. Introduction

Chronic activation of cardiomyocyte surface receptors such as those for epinephrine, endothelin-1, prostaglandin $F_{2\alpha}$, and angiotensin II causes a series of events that culminate in myocyte hypertrophy and contractile dysfunction (reviewed in [1]). A common feature of these receptors is that they couple to the G_q class of heterotrimeric G proteins, suggesting that activation of G_q signalling by these agents represents a common point of convergence which is necessary to evoke the phenotype. Recently we have further explored this notion at the biochemical and physiological level by overexpressing the α subunit of G_q in the hearts of transgenic mice [2]. The mice develop cardiac hypertrophy, depressed ventricular contractility at baseline and in response to β-adrenergic receptor (βAR) agonists, diminished basal and agonist stimulated adenylyl cyclase activity, and activation of a fetal cardiac gene program with increases in atrial natriuretic factor (ANF) and β-myosin mRNA [2].

Recent studies have delineated several potential mechanisms of βAR dysfunction in these mice which are associated with ventricular dysfunction and βAR unresponsiveness in vivo [2,3]. These include an uncoupling of the βAR from G_s, an increase in $G_{\alpha i}$, and a decrease in expression and/or function of adenylyl cyclase. In contrast to other mouse models of hypertrophy [4], the $G_{\alpha q}$ mouse does not exhibit changes in expression of βAR or the βAR kinase [3]. The decrease in adenylyl cyclase expression and catalytic activity, which amounts to ∼45% loss of type V adenylyl cyclase, is particularly intriguing since adenylyl cyclase expression has been reported to constrain βAR signalling in rat neonatal myocytes [5]. Furthermore, adenylyl cyclase gene expression or function is depressed in several other animal models of heart failure [6–8]. Because the cardiac hypertrophy and systolic dysfunction in the $G_{\alpha q}$ mouse are associated with impaired βAR signalling which may be multifactorial (at the level of the receptor, G protein and adenylyl cyclase) we have sought to identify cause-and-effect relationships at each level and to identify the ramifications of correcting each biochemical lesion. In so doing, an assignment of a molecular basis for the various facets of the $G_{\alpha q}$ phenotype can be made. In this report, we have overexpressed type V adenylyl cyclase in transgenic mice to a level that would be expected to achieve replacement when mated to $G_{\alpha q}$ mice. These transgenic mice displayed enhanced catalytic activity consistent with functionally overexpressed adenylyl cyclase and displayed no deleterious consequences from such overexpression. The type V adenylyl cyclase (ACV) transgenics were then mated to the $G_{\alpha q}$ overexpressers, and the biochemical and physiologic consequences were investigated.

2. Materials and methods

2.1. Transgenic mice

Transgenic mice of the FVB/N background which overexpress $G_{\alpha q}$ ∼5 fold over background ($G_{\alpha q}$40) have been previously described [2] and are hereafter referred to as $G_{\alpha q}$ mice. To create the ACV over-

*Corresponding author. Fax: (1) (513) 558-0835.
E-mail: stephen.liggett@uc.edu

Abbreviations: βAR, β-adrenergic receptor; $G_{\alpha q}$, α subunit of the G_q guanine nucleotide binding protein; ANF, atrial natriuretic factor; ACV/$G_{\alpha q}$, heterozygous crosses of transgenic mice overexpressing $G_{\alpha q}$ and type V adenylyl cyclase

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expressers the rat cDNA for type V adenylyl cyclase (obtained from R. Premont, Duke University) was digested with *Xho*I releasing a fragment consisting of 434 bp of 5′ untranslated sequence, followed by the 3787 bp of the coding region sequence and 231 bp of the 3′ untranslated sequence. This was ligated into the *Sal*I site of the full length α-myosin heavy chain (αMHC) promoter construct. The integrity of the construct was confirmed by sequencing. DNA for microinjection was liberated from the αMHC-ACV plasmid by *Bam*HI digestion and microinjection into male pronuclei of FVB/N embryos was carried out by standard procedures.

2.2. Transgene identification

Pups were screened via Southern analysis using *Eco*RI digested genomic DNA extracted from tail clips. The probe used to detect presence of the transgene was a ^{32}P labeled (random priming method) 4 kb *Eco*RI fragment of the αMHC/ACV plasmid consisting of ∼1.9 kb of the αMHC promoter and ∼2.1 kb of the type V cDNA. A heterozygous line (designated 14.3) which expresses ACV ∼50% over background non-transgenic levels was chosen for mating to heterozygous $G_{\alpha q}$ overexpressing mice. The presence of the two transgenes was determined by Southern blot using the above probe and a previously described $G_{\alpha q}$ probe [2]. First and second generation double (denoted ACV/$G_{\alpha q}$) and single transgenic and non-transgenic littermates, at ages 12–16 weeks, were used for all studies.

2.3. Adenylyl cyclase activities

Ventricles were homogenized with a Polytron for 10 s in cold 5 mM Tris (pH 7.40), 2 mM EGTA buffer containing the protease inhibitors (5 μg/ml) leupeptin, PMSF, soybean trypsin inhibitor, benzamidine and aprotinin. Homogenates were centrifuged at 500×*g* for 10 min at 4° and the pellet discarded. The supernatant was centrifuged at 40 000×*g* for 10 min and the pellet resuspended in a buffer that provided for a final concentration in the adenylyl cyclase reaction of 2 mM Tris, 4.8 mM $MgCl_2$, 0.8 mM EGTA, pH 7.40, with the aforementioned protease inhibitors. Adenylyl cyclase activities were measured essentially as previously described [3]. 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 3×10^6 dpm [α-^{32}P]ATP. Reactions were carried out for 10 min at 37°C and contained various concentrations of isoproterenol or 100 μM forskolin. Reactions were stopped by dilution with 1.0 ml of a 4° solution containing excess ATP and cAMP, and 25 000 dpm/ml [^{3}H]cAMP used to determine column recovery. [^{32}P]cAMP was separated by chromatography over alumina columns.

2.4. Radioligand binding

For determination of total βAR density, reactions consisted of membranes (∼50 μg) and 400 pM ^{125}I-CYP in the absence and presence of 1.0 μM alprenolol, used to define non-specific binding [9]. Binding reactions were carried out for 2 h at 25°C and terminated by dilution and rapid filtration over GF/C (Whatman) filters. Specific binding, defined as total minus non-specific, was normalized to protein, and is expressed as fmol/mg.

2.5. Northern blots

Total RNA was extracted from cardiac tissue using Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH) and the manufacturer's protocol. Cardiac gene expression was compared in non-transgenic and transgenic animals using RNA dot blotting and gene specific antisense oligonucleotides [10], as previously described [2]. Briefly, 2 μg of RNA was denatured and blotted to Hybond N$^+$ membranes. After blotting, membranes were prehybridized for 1 h at 55°C in a solution containing 5×SSC, 0.5% SDS, 1×Denhardt's, and 90 μg/ml salmon sperm DNA. Samples were then hybridized with ^{32}P labeled DNA probes for 5 h at 55°C and washed. Gene expression was quantitated using a PhosphorImager and ImageQuant Software. Signal intensities for each dot were normalized to GAPDH to account for small differences in loading.

2.6. Echocardiography

Echocardiography was performed in mice sedated with intraperitoneal Avertin (1.0 ml/g of a 2.5% solution) as described [2]. Fractional shortening (FS) was calculated from the end-systolic and end-diastolic dimensions (ESD and EDD, respectively) by the formula: FS = (EDD−ESD)/EDD.

2.7. Histopathology

Following dissection, whole hearts from 12 week old mice were fixed in 10% neutral buffered formalin for 24 h and dehydrated in 70% ethanol. Hearts were embedded in paraffin and sectioned in the long axis. Sections 4 μm in thickness were stained with hematoxylin and eosin or Masson's trichrome stain and examined microscopically.

2.8. Statistical analysis

Results from studies were compared by paired or unpaired *t*-tests as appropriate. P values < 0.05 were considered significant. Curve fitting of dose-response data was carried out by an iterative least squares technique using software from GraphPad (San Diego, CA). Data are presented as mean ± S.E.M. of the indicated number of independent experiments, each performed with a different mouse.

3. Results and discussion

We have hypothesized that the reduction in adenylyl cyclase expression and function in the $G_{\alpha q}$ mice could sufficiently alter the stoichiometric equilibrium between receptor, G_s, and adenylyl cyclase such that βAR signalling is impaired. Thus, we bred the $G_{\alpha q}$ mice with a relatively low overexpresser of ACV in order to achieve replacement levels of the cyclase. The ACV/$G_{\alpha q}$ mice maintained an ∼5 fold increased expression of $G_{\alpha q}$ in the heart (data not shown). Forskolin stimulated adenylyl cyclase activities were determined in cardiac membrane to assess function of the type V adenylyl cyclase transgene (Fig. 1). These activities were ∼50% lower in the $G_{\alpha q}$ mice compared to non-transgenic littermates (273 ± 68 vs. 533 ± 91 pmol/min/mg, $n = 4$, $P < 0.005$). The ACV/$G_{\alpha q}$ crosses had forskolin stimulated activities (819 ± 160 pmol/min/mg) that were clearly increased over the $G_{\alpha q}$ mice ($P < 0.02$) and were indeed somewhat higher than those of non-transgenic littermates ($P = 0.04$, Fig. 1). As previously reported $G_{\alpha q}$ mice do not display a downregulation of cardiac βAR expression [2]. The type V adenylyl cyclase transgenics and the ACV/$G_{\alpha q}$ crosses also had βAR expression levels that were no different from those of non-transgenic littermates (25 ± 5.8 and 28 ± 4.9, vs. 28 ± 3.2 fmol/mg, $n = 3$–5, P = NS). Basal and isoproterenol-stimulated activities are

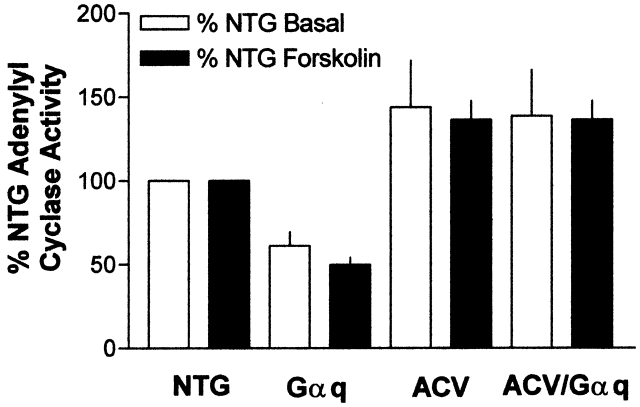

Fig. 1. Functional properties of the ACV transgene. Cardiac membranes were prepared from non-transgenic (NTG), heterozygous $G_{\alpha q}$ overexpressing transgenic mice, heterozygous ACV overexpressing transgenic mice, and dual heterozygous ACV/$G_{\alpha q}$ mice. Maximal catalytic adenylyl cyclase activity was determined by incubation with 100 μM forskolin. Data are normalized to the NTG response. $G_{\alpha q}$ hearts have ∼50% decreases in basal and forskolin stimulated adenylyl cyclase activities. Transgenic expression of ACV resulted in an enhanced stimulation which is not counterregulated in the ACV/$G_{\alpha q}$ mice. Results are from five independent experiments.

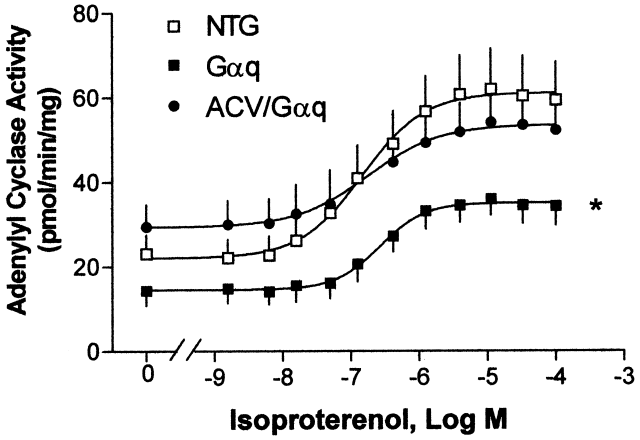

Fig. 2. βAR stimulated adenylyl cyclase activities are restored in G_{aq} mice by transgenic replacement of ACV. Cardiac membranes were prepared and activities determined in response to the indicated concentrations of the β-agonist isoproterenol. Both basal and maximal isoproterenol stimulated activities were depressed in the $G_{\alpha q}$ mice compared to NTG. The EC_{50} for isoproterenol was also increased (see text for results). The response from ACV/$G_{\alpha q}$ cardiac membranes was indistinguishable from NTG. Results are from five independent experiments. *Overall response different from NTG, $P < 0.01$.

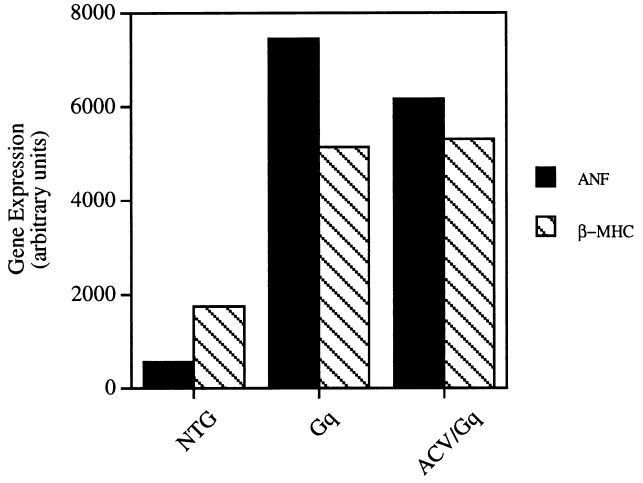

Fig. 4. Genetic markers of hypertrophy are not altered by ACV restoration in $G_{\alpha q}$ mice. RNA was extracted from the hearts of the indicated mice and expression of transcripts for ANF and β-myosin heavy chain (β-MHC) determined. Results are means from four mice per line, normalized to the group mean expression of GAPDH.

shown in Fig. 2. Basal adenylyl cyclase activities were reduced in the $G_{\alpha q}$ transgenic mice compared to non-transgenic littermates (14.5 ± 3.6 vs. 23.2 ± 4.4 pmol/min/mg). Basal activities of the ACV/$G_{\alpha q}$ crosses were 29.5 ± 5.3 pmol/min/mg, which were not statistically different from non-transgenic basal activities. Maximal isoproterenol stimulated activities of the $G_{\alpha q}$ mice were lower (34.4 ± 4.6 vs. 59.5 ± 8.9 pmol/min/mg) and the EC_{50} values higher (270 ± 20 nM vs. 149 ± 7 nM, $P < 0.005$) as compared to non-transgenics (NTG). The ACV/$G_{\alpha q}$ crosses revealed a maximal response of 52.2 ± 6.7 pmol/min/mg, which was not different from that of non-transgenic littermates. The EC_{50} for this response was 209 ± 64 nM ($P = 0.19$ compared to NTG). So from these data it appears that βAR sensitivity and maximal responsiveness were fully restored in $G_{\alpha q}$ mice by replacement of type V adenylyl cyclase. Since an increase in G_i is another potential mechanism for the $G_{\alpha q}$ phenotype, and it is conceivable that increased adenylyl cyclase could regulate G_i, we assessed $G_{\alpha i3}$ levels in the hearts by Western analysis. The levels of $G_{i\alpha 3}$ were identical in extracts from $G_{\alpha q}$ and ACV/$G_{\alpha q}$ mice (data not shown).

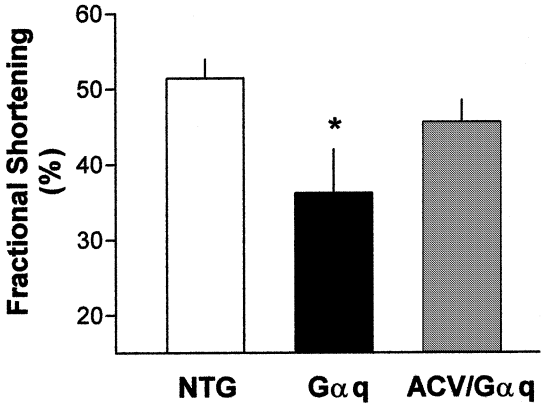

Fig. 3. Ventricular function is improved in ACV/$G_{\alpha q}$ mice. Echocardiograms were performed on the indicated transgenic mice. The $G_{\alpha q}$ phenotype is depressed fractional shortening and decreased heart rate compared to NTG mice. When ACV is coexpressed (ACV/$G_{\alpha q}$ mice) fractional shortening and heart rates are increased and not statistically different from those of NTG mice. Results are from 5–6 mice in each group. *$P < 0.01$.

Ventricular function was assessed by echocardiography (Fig. 3). Consistent with our previous study [2], both end-systolic and end-diastolic dimensions were increased in $G_{\alpha q}$ mice and left ventricular fractional shortening was decreased (36 ± 6% as compared to 51 ± 2% for non-transgenic littermates, $P < 0.05$). In the ACV/$G_{\alpha q}$ crosses, end-systolic and end-diastolic dimensions were improved and the fractional shortening was increased to 46 ± 3%, which is not statistically different from that found with non-transgenic littermates ($P = 0.27$). As indicated, the $G_{\alpha q}$ mice have lower heart rates compared to non-transgenic littermates (286 ± 28 vs. 429 ± 25 bpm). The ACV/$G_{\alpha q}$ crosses had intermediate heart rates of 388 ± 13 bpm which were not different from those of NTG ($P = 0.30$).

In the $G_{\alpha q}$ model of hypertrophy, we have also extensively examined the cardiac expression of several fetal genes [2]. Of the large assay initially assessed, levels of ANF and β-myosin transcripts were substantially increased in the $G_{\alpha q}$ mice compared to non-transgenic littermates, and we have found these to be sensitive indicators of improvements in hypertrophy [3]. The results of such RNA dot blot studies are shown in Fig. 4. The $G_{\alpha q}$ mice displayed 13.0 and 2.9 fold increases in expression of ANF and βMHC respectively. Co-expression of type V adenylyl cyclase, which normalized βAR signalling and improved cardiac function, does not appear to have a significant effect on ANF and βMHC gene expression (11.0 fold and 3.0 fold increased, respectively). Thus, based on these molecular markers, hypertrophy persisted despite improved function. Heart to body weight ratios confirmed these findings. The $G_{\alpha q}$ mice had enlarged hearts with ratios (mg/g) of 11.3 ± 1.0 compared to 8.2 ± 1.0 for NTG ($P = 0.03$). ACV/$G_{\alpha q}$ mice showed no decrement in this ratio (11.1 ± 1.7). Histopathologic studies of $G_{\alpha q}$ mice showed hypertrophic myo-

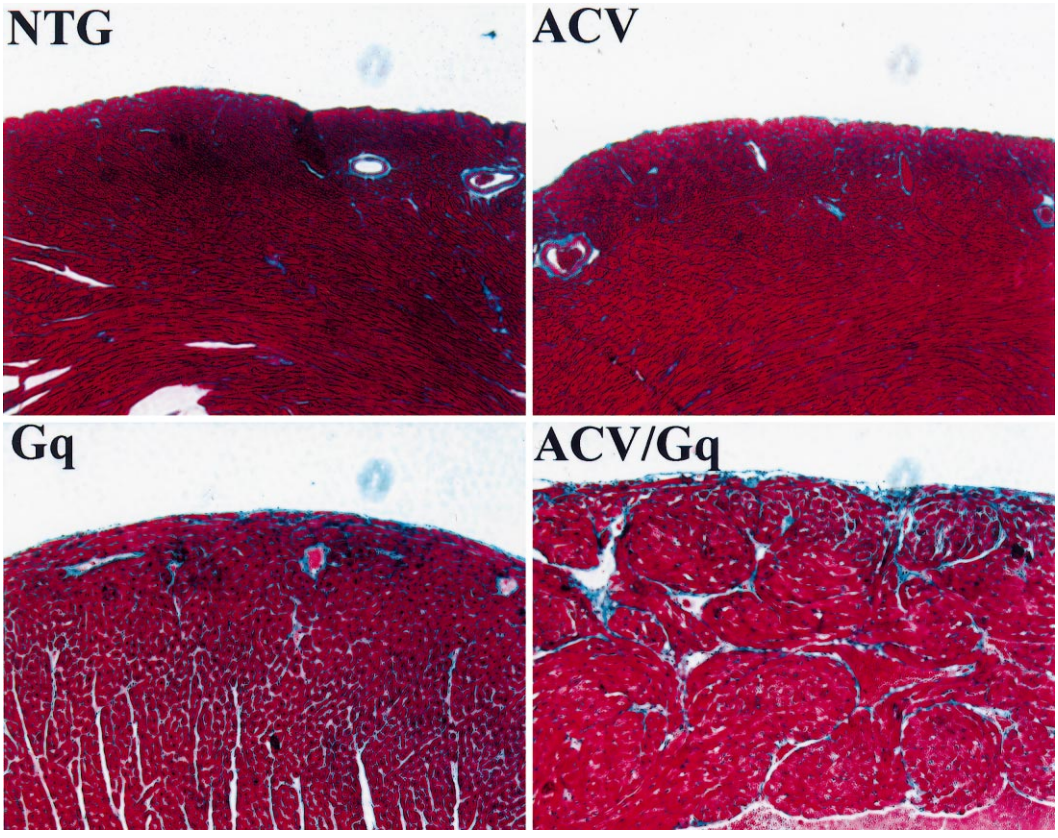

Fig. 5. Cardiac histology. Shown are 4 μm sections of left ventricles stained with Masson's trichrome of NTG, ACV, $G_{\alpha q}$ and ACV/$G_{\alpha q}$ mice. Magnification is 100×.

cytes without inflammation or a consistent degree of significant fibrosis; ACV/$G_{\alpha q}$ mice showed no improvement in the hypertrophy with clear areas of fibrosis (Fig. 5).

In the current studies we have shown that a significant component of the signalling defect of βAR stimulation of adenylyl cyclase in the $G_{\alpha q}$ overexpressing model of hypertrophy and ventricular dysfunction is a decrease in the expression/function of cardiac type V adenylyl cyclase. Under the conditions of $G_{\alpha q}$ overexpression, which evokes a series of pathologic sequelae that includes βAR dysfunction, we have found several other potential mechanisms which may impair βAR function. βAR physical coupling to $G_{\alpha s}$, as determined in agonist competition studies in the absence of guanine nucleotide, is decreased in cardiac membranes from $G_{\alpha q}$ overexpression. In addition, $G_{\alpha i2}$ and $G_{\alpha i3}$ levels are increased. While any of these two, or the decrease in adenylyl cyclase, could theoretically result in βAR dysfunction, explicit testing to delineate the predominant mechanism is necessary. Given that the stoichiometry of βAR to G_s to adenylyl cyclase in rat myocytes has been reported [5] to be 1:200:3, a decrease in the latter component, as is seen in the $G_{\alpha q}$ overexpressing mouse, has the *potential* to limit βAR signalling. We have recently reported the results of overexpressing the β_2AR in the hearts of $G_{\alpha q}$ mice [3]. A ∼30 fold overexpression failed to improve isoproterenol stimulated adenylyl cyclase activities. With ∼140 fold overexpression partial restoration of adenylyl cyclase responsiveness was observed. These data suggested that alterations of a 'downstream' element of the signal transduction cascade has a significant effect on βAR signalling. In the current report we show that a relatively small increase in type V adenylyl cyclase, which essentially restores catalytic function of the enzyme to normal (non-transgenic) levels, results in full rescue of βAR stimulation of adenylyl cyclase. Taken together, then, the evidence indicates that decreases in type V adenylyl cyclase have a major impact on βAR signalling in this physiologically relevant mouse model. Such modulation appears to have a direct impact on ventricular function as well. Rescue of βAR signalling to adenylyl cyclase was accompanied by significant improvement of ventricular function when type V adenylyl cyclase levels were restored. However, such improvement was not accompanied by changes in hypertrophy. Thus the stimulus for development of hypertrophy in the $G_{\alpha q}$ mouse appears to be independent of the decrease in βAR/cAMP signalling. Interestingly, a prolonged decrease in sympathetic stimulation to the heart may actually serve a protective effect against worsening failure [11]. Thus any gene therapy resulting in enhanced cardiac βAR function may be best suited for the acute, rather than chronic, setting.

Our experimental design differs markedly from that of Roth et al. [12] who have reported the effects of ∼20 fold overexpression of type VI adenylyl cyclase in the heart. Type V and VI isoforms differ in a number of key aspects [13–15]. Furthermore, based on studies in chick heart, type VI adenylyl cyclase is thought to be minimally, if at all, expressed in the cardiomyocyte [16]. Thus the aforementioned studies utilized overexpression of a non-native cyclase to high levels. This then likely represents 'forced' improvement without identification of a biochemical lesion per se. On the other hand, we have expressed the dominant adenylyl cyclase isoform of the

cardiomyocyte to a level that when these transgenic mice are crossed with $G_{\alpha q}$ mice, a restoration of this isoform to non-transgenic levels is attained. In this way the mechanism of βAR dysfunction in the $G_{\alpha q}$ mouse could be directly explored.

In conclusion, there are several potential mechanisms that cause a decrease in βAR signalling and depressed ventricular function in $G_{\alpha q}$ overexpressing transgenic mice. One of these, a decrease in type V adenylyl cyclase, has been explicitly tested by creating dual transgenic mice overexpressing the cyclase and $G_{\alpha q}$. Our findings indicate that the decrease in expression of type V adenylyl cyclase appears to be the major basis for this defective βAR signalling, since restoration of expression via transgenesis normalizes βAR signalling and ventricular function.

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